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CLINICAL AND EXPERIMENTAL

THE DEVELOPMENT OF HISTIOCYTES AND MACROPHAGES FROM LYMPHOCYTES

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HISTIOCYTES are the active, ameboid, dye-storing macrophages or potential macrophages of the hematopoietic organs and connective tissues believed by Aschoff and Kiyono, who named them, to be tissue cells not related in any way to lymphocytes or other blood cells. Although they appear in various forms, depending on their activities and environmental relations, they are included with the cells of the reticuloendothelial system because of their storage and phagocytic capacity. The term "histiocyte" usually refers to the free, rounded or ameboid cells which may become very numerous in inflammatory exudates of connective tissue and serous cavities. They are the *polyblasts* of Maximow, distinguished from lymphocytes in both dry smears and tissue sections by paler and more granular and vacuolated cytoplasm, and by the thinner nuclear membrane, and the more diffuse and characteristic chromatin pattern resembling that of the fixed and free reticular cells of the hematopoietic organs. The free reticular cells of lymph nodes and spleen resemble the active histiocytes of the connective tissues and serous cavities. Their number is greatly increased in these situations by any acute inflammatory condition. In the lymphoid organs the increase is due largely to their mobilization from the network of fixed reticular cells, in the connective tissue to activation of the resting or inactive histiocytes (clasmatocytes, perithelial cells, adventitial cells), and to a slight extent to mitosis. While local origin in connective tissue accounts for many of the increased number of histiocytes, it does not explain the appearance of an excessive number of these cells during a severe acute inflammatory reaction.

Metchnikoff, 1892 and later, showed that lymphocytes and monocytes may migrate from blood vessels and transform to "macrophages" in both septic and aseptic inflammation. Lithium carmine (Ribbert[31]), pyrrhol blue (Goldmann[14, 15]), and other acid electronegative

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colloidal dyes proved to be very useful in later experiments in which transitions from lymphocytes to dye-storing macrophages were observed by many investigators.

Objections to the conclusions from these experiments resulted from the publication in 1914 by Kiyono,[17] working in Aschoff's laboratory, of his very extensive experiments with lithium carmine. The dye-storing cells were termed "histiocytes" because both he and Aschoff believed them to be of exclusive tissue origin. Aschoff[1] later incorporated them in his *reticuloendothelial system.* In 1919 Kiyono,[18] with Nakanoin, reversed his earlier concept and agreed that lymphocytes may transform to "histiocytes" and active macrophages, although Aschoff,[1, 2] Seemann,[33, 34] and others still believed in the independence of the histiocytes.

The transformation stages have been adequately demonstrated in the long series of beautifully illustrated studies of Maximow[21-25] and Bloom[5-9] of inflammatory reactions, and tissue cultures of blood-forming organs, blood, lymph, and serous exudates. For those who demand proof for such transformations as seen in imprints and dry smears, the experiments of Kolouch,[19] Rebuck,[29, 30] and Berman[4] are cited.

Kolouch, using young rabbits, made subcutaneous injections of egg albumen in several places in each animal, followed by serial biopsies of the inflamed loose connective tissue. The tissue was spread on slides and dried, and then stained with May-Grünwald-Giemsa as for blood smears. A good series of photographs shows the transformation stages of both clasmatocytes and emigrated lymphocytes. The histiocytes and macrophages derived from both sources are easily distinguished in the earlier stages but cannot be separated in the later stages. The change of the nuclear pattern of the lymphocytes to that of the diffuse chromatin structure of the histiocytes can be seen in these photographs.

Rebuck scraped small patches of epithelium from his own arm and bound cover glasses to the denuded areas. Many of the lymphocytes and monocytes migrating to the inflamed areas adhered to the cover glasses, which were removed at varying intervals, dried quickly, and stained as for blood smears. These preparations provided clear-cut demonstrations of the transitional stages between lymphocytes and histiocytes (polyblasts) and active macrophages.

From the foregoing, and from my own experiments,[10-13] and from the study of histoplasmosis by Watson,[37] it seems that the evidence in favor of the potentiality of lymphocytes to transform to polyblasts and macrophages is complete, and that in the later stages of this transformation these cells cannot be distinguished from those derived from the local fixed histiocytes. These transformations occur as a phase of inflammatory reactions; they also are seen in the normal organism, although less frequently. The transformation stages are most numerous in the early stages of inflammatory reactions of loose connective tissue, and in tissue cultures of blood, lymph, and lymph nodes. The type of irritant used for the inflammatory reactions seems of little importance as long as it is not too toxic.

There is some difference of opinion regarding the ability of lymphocytes to develop to histiocytes in situ during inflammatory reactions of lymphoid organs. Latta and Schulz,[20] using various colloidal dyes and India ink in rats, described stages between large lymphocytes and macrophages in sinuses and at the margins of nodules in sections of lymph nodes. Reticular cells also produced macrophages which, in the later stages, could not be distinguished from those derived from lymphocytes. Taliaferro and Mulligan[36] noted intense macrophage formation in spleens of monkeys infected with malaria, and concluded that the spleen, through its macrophages, was the chief organ of defense against the parasites. There was marked lymphoid hyperplasia with large follicles from which lymphocytes migrated to the pulp and transformed to macrophages. Free reticular cells also formed macrophages, but not as many as were derived from lymphocytes.

Maximow's student, Babkina,[3] studied aseptic inflammation resulting from the insertion of celloidin tubes into lymph nodes, spleen, and marrow of 39 adult rabbits. In lymph nodes the change of lymphocytes to polyblasts was very slow and occurred only in the later stages. Maximow's explanation[25] of this slow reaction was that lymphocytes of the lymphoid organs are too immature, and so must be conditioned by circulation in the blood and migration to the tissues before the change can occur, except in very severe and persistent inflammatory reactions

of the organs. Such a reaction was noted by Bloom[6] in inflammation of a lymph node of a rabbit inoculated with a virulent strain of *B. monocytogenes*. In the wall of an abscess formed in this node there were numerous transitions between small and medium-sized lymphocytes and polyblasts, as shown clearly in his illustration.

The conditions governing the transformation of lymphocytes to histiocytes in vivo still constitute an interesting problem, as is evident from the foregoing brief summary of some of the relevant literature. It was decided to study the problem in animals injected aseptically with the relatively mild irritants, lithium carmine, pyrrhol blue, egg white, and egg yolk, which would induce inflammatory reactions less severe than those resulting from Babkina's experiments, and the experimental infections with malaria parasites and *B. monocytogenes* of Taliaferro and Mulligan, and Bloom.

MATERIAL AND METHODS

The following material was examined for evidence of the transformation of lymphocytes to histiocytes and macrophages especially in lymph nodes and spleen.

(a) Omentum and imprints and sections of mesenteric lymph node of a rabbit which received 3 intravenous and 3 intraperitoneal injections of 5 ml. of a 1 per cent solution of lithium carmine.

(b) Omentum of rabbit after one intraperitoneal injection of typhoid toxin.

(c) Peritoneal exudate and sections of lymph node, spleen, and fat lobule of a rat which had received 4 intraperitoneal injections of 3 ml. of 1 per cent pyrrhol blue. The animal was killed 24 hours after the last injection.

(d) Smears of human tubercular peritoneal exudate.

(e) A series of 6 rabbits used for other experiments in collaboration with Dr. Berry Campbell.

Four of these rabbits were injected with egg yolk into the left leg. One rabbit received 3 intravenous and left-foot injections of egg white in Sims's solution, and one intraperitoneal injection. The same solution was injected once into the left foot and peritoneal cavity of the sixth rabbit.

Smears were made of the peritoneal exudate of the 2 rabbits which were given intraperitoneal injections. Right and left lumbar and popliteal nodes were removed from the rabbits receiving egg yolk. Popliteal and mesenteric lymph nodes and spleens were taken from the last two rabbits. Fixation was in Helly's solution after imprints had been made. Imprints of organs and smears of peritoneal exudates were stained with Wright's stain as for blood smears. Sections were stained with Delafield's hematoxylin or Dominici's eosin-orange G-toluidin blue.

OBSERVATIONS

Omentum.—The omentum of the rabbit which received intraperitoneal injection of typhoid toxin is an excellent preparation for demonstrating the transformation of lymphocytes to histiocytes. Histiocytes of various sizes with eccentric nuclei are very numerous in all parts of the spread. Many small lymphocytes are present, even in the thinner areas. They are much more numerous than in the omentum of a normal animal, and there are many transitions between them and the small and larger histiocytes. The first change in the lymphocyte seems to be lobulation of the nucleus, scattering of chromatin, and thinning of the nuclear membrane before there is much increase in the size of nucleus or cytoplasm. The nucleus becomes eccentric, and has the characteristic

histiocytic chromatin pattern when the cell reaches the larger size. In some cells the eccentric nucleus has retained the structure of the small lymphocyte nucleus while the cytoplasm has increased. Many large "pus corpuscles" of a late abscess are cells of this type.

Heterophil leukocytes also are numerous in this omentum and the vessels are gorged with blood. It seems likely that many of the lymphocytes have migrated from the blood. They, and most of the scattered large histiocytes, could also have come from the tache laiteuses and perivascular lymphoid sheaths where lymphocytes and histiocytes are numerous in the normal omentum. It seems that in this type of severe inflammation the normal number of histiocytes present cannot meet the demand for active mobilized polyblasts, and that this explains the development of many of them from lymphocytes. The reaction is similar to what occurs in loose connective tissue in similar circumstances when fixed and resting histiocytes (clasmatocytes) and emigrated lymphocytes give rise to active polyblasts whose origin cannot be determined in the later stages.

In the rabbit which received the intraperitoneal and intravenous injections of lithium carmine the inflammatory reaction of the omentum is less severe than in the animal which received typhoid toxin. Most of the free histiocytes contain dye granules; some are loaded with granules, while others contain only a few. Here the developmental stages from lymphocyte to histiocyte are easily followed because, as the lymphocytes increase their cytoplasm and rearrange their nuclear pattern, they begin to deposit dye granules. However, the transitional stages are not nearly as numerous as they are in the typhoid experiment, indicating that the demand for mobilized histiocytes is supplied largely from the histiocytes present before the first carmine injection.

Peritoneal Exudates.—The peritoneal exudate of the rat which received 4 injections of pyrrhol blue contains numerous histiocytic cells with dye granules. Most of these cells must have entered the peritoneal cavity as histiocytes, as lymphocytes are scarce and only an occasional one of them shows evidence of histiocytic change with beginning dye storage.

According to Schott[32] the normal rat and rabbit do not have lymphocytes in their peritoneal transudate, although they are present in the guinea pig. In the rat, following intraperitoneal injection of foreign erythrocytes, Schott found that most of the macrophages were derived from fixed connective tissue cells, while a small number originated from scarce emigrated lymphocytes. This reaction evidently was similar to that which follows the injection of colloidal dye.

Of the two rabbits receiving intraperitoneal injections of egg albumen the one receiving its injection twenty-one days after the first of two intravenous injections of the same substance showed the most numerous lymphocytes, and transitions between them and histiocytes in its exudate. Lymphocytes, and some intermediate stages between them and macrophages, were present twenty-four hours after the injection, but were more numerous at forty-eight hours. A few lymphocytes were still present at seventy-two hours, and an occasional one of these showed histiocytic changes.

If Schott[32] is correct in stating that the normal rabbit's peritoneal transudate does not contain lymphocytes, it must be assumed that, in these experimental rabbits, the lymphocytes have emigrated to the peritoneal cavity where they transformed to macrophages. When demand for macrophages is satisfied, emigration of lymphocytes ceases.

The conclusion from these experiments is that most of the numerous macrophages have been derived from mobilized histiocytes in the omentum and elsewhere, while a few of them have originated from transformed lymphocytes.

In the human tubercular exudate the reaction is largely of the histiocytic-monocytoid cell type. Only a few lymphocytes are present, but even here an occasional one shows beginning histiocytic change. The monocytoid cells are strikingly similar to monocytes of normal blood. According to Schilling,[31a] such cells are genuine monocytes derived from fixed histiocytoid cells by way of the free histiocyte. However, various types of exudates show that the free histiocytes also may be derived from lymphocytes, as in inflamed connective tissue. Whether the free histiocytes persist as histiocytes or become macrophages or monocytoid cells depends on the type of experiment or pathology involved.

Lymph Nodes and Spleens.—In the mesenteric lymph node of the rabbit receiving lithium carmine there are many "dye cells" with the usual distribution. Many of them are free, rounded histiocytes with eccentric nuclei occurring in sinuses and margins of follicles. Others are in large groups in medullary cords and marginal portion of follicles. There is no regularity in the distribution of the dye cells, especially of the free ones. Some portions of the sections contain very little dye, although the fixed reticular cells usually have some.

In the imprints of this node, lymphocytes that were changing to dye cells could not be detected, but a few such instances were noted in the sections after prolonged search. A few large histiocytes loaded with dye granules had eccentric nuclei of size and structure of small lymphocyte nuclei.

In this material the demand for dye cells (macrophages) was supplied chiefly by the histiocytes present before injection, or mobilized from the fixed reticulum in response to the irritation caused by the dye. Lymphocytes that show any indication of histiocytic development are very scarce.

Sections of a lymph node of the rat injected with pyrrhol blue have a few lymphocytes showing beginning histiocytic transformation and dye storage. They are located in the sinuses where there is a mixture of lymphocytes and histiocytes. The spleen of this animal also shows a few small histiocytes with nuclei of small lymphocytes but with a slightly increased amount of cytoplasm containing a few dye granules.

Dye cells are very numerous under the thin capsule of a fat lobule where histiocytes are often numerous in a normal animal. A few small lymphocytes are to be seen among them, and an occasional one of these shows histiocytic transformation. The intermediate cells are very scarce in all three of these organs from the experimental rat, and it requires patient searching to find them.

Transitions between lymphocytes and histiocytes could not be detected in any of the lymph nodes and spleens of the rabbits treated with egg yolk or egg white. Evidence of inflammatory reaction of varying degrees of intensity was

noted in most of the nodes, especially in the left lumbar and popliteal nodes of the animals injected with yolk in the left leg. Some nodes of these 4 animals are characterized by increase of reticulum, or of free reticular histiocytes in the sinuses. In other nodes the number of large basophilic "reticular lymphocytes" is increased, and this is correlated with reduction in the number of histiocytes in the sinuses. The nodes are of normal structure except for the changes indicated.

The lymph nodes of the rabbits injected with egg white also show an increase of reticulum and of free histiocytes in the sinuses. In the animal receiving a single intraperitoneal and left foot injection there are many more large basophilic lymphocytes in the left popliteal node than in the right node.

Plasma cells are numerous in the spleen of the rabbit that received repeated injections. A few plasma cells also occur in the right popliteal node of this animal. Plasma cells are scarce in spleen and nodes of the other animal.

There was no evidence for the development of histiocytes from lymphocytes in spleen or nodes of these animals. With this exception the results of these experiments with the rabbits are not important for the problem at hand, and for this reason the finer details of the experiments have not been listed.

DISCUSSION

The peritoneal exudate of the rabbit that received one injection of egg white furnishes a good example of the extent to which lymphocytes are involved in the cellular composition of an exudate in the early stages of its formation. Cellular migrations and transformations are followed with less difficulty in exudates of serous cavities than in connective tissue during inflammatory reactions.

In the rat, lymphocytes are scarce twenty-four hours after a fourth injection of pyrrhol blue, but dye cells are numerous. As an occasional one of the scarce lymphocytes showed histiocytic transformation, it was assumed that lymphocyte emigration was more extensive after the earlier injections and that those lymphocytes gave rise to some of the numerous histiocytes. It seems that after the fourth injection the histiocytes resulting from the earlier injections have disposed of most of the additional dye, and that few lymphocytes are required for the production of new dye cells.

An occasional one among the scarce lymphocytes present in the human tubercular exudate examined showing histiocytic development indicates that some of the very numerous histiocytes of this exudate have been derived from lymphocytes. Tubercular exudates usually are characterized by a high percentage of lymphocytes. It seems, therefore, likely that this exudate is in a very late stage of its formation and that most of its lymphocytes have developed to histiocytes. Cytologically the exudate resembles closely the late stages of the tissue exudates obtained by Rebuck[29] in his human "window" experiments.

The omentum of the carmine rabbit and of the rabbit receiving typhoid toxin intraperitoneally furnished the opportunity to study a relatively mild inflammatory reaction in a tissue compared to one of much greater intensity. In the latter the number of emigrated lymphocytes and transitional stages between

them and the very numerous histiocytes is far greater than in the former. However, in general character the two reactions are similar.

Lymphocytes showing any indication of transforming to histiocytes are very scarce in the mesenteric lymph node of the carmine rabbit, and in lymph nodes and spleen of the pyrrhol rat. Fixed and free dye cells are numerous in both cases, and it is evident that the great majority of the free dye cells are of reticular origin. The reticulum is able to supply most of the macrophages needed for disposal of the dye.

Not a single instance of histiocytic transformation of lymphocytes could be found in lymph nodes and spleens of the rabbits injected with egg white and egg yolk, although there was some reaction of the reticulum in most of these organs. In some cases this resulted in an increased number of reticular lymphocytes, or large lymphocytes in addition to free histiocytes.

In these more or less acute reactions of the lymphoid organs the reticulum was able to take care of foreign matter, so there was no necessity for the production of macrophages from lymphocytes. The organs of these animals were not subject to persistent intense irritation, as in the malaria experiments of Taliaferro and Mulligan[36] and the celloidin tube experiments of Babkina,[3] which resulted in the formation of many histiocytes and macrophages from lymphocytes.

These experiments have shown again that lymphocytes emigrating to connective tissue and peritoneal exudates during inflammatory reactions may transform to histiocytes and macrophages. The extent of the emigration of lymphocytes and their development to histiocytes depends on the severity of the inflammatory reaction, and on the ability of the involved tissues to supply histiocytes from their own cells having histiocytic potencies, such as the numerous clasmatocytes of the omentum and the reticulum of lymphoid organs.

The failure of all but a few lymphocytes of lymphoid organs to transform to histiocytes in acute conditions seems not to be due to immaturity of the cells, or necessity for conditioning by circulation in the blood (Maximow[25]), but rather to the phagocytic- and histiocyte-producing potency of the reticulum which disposes of foreign material before the lymphocytes become affected by it. On the other hand, some severe inflammatory reactions persisting for a longer period may include extensive involvement of the lymphocytes.

SUMMARY

Transitional stages between lymphocytes and histiocytes were numerous in the omentum of a rabbit following intraperitoneal injection of typhoid toxin. Lithium carmine caused a milder inflammatory reaction with fewer lymphocytes and transitional stages.

Developmental stages from lymphocytes to histiocytes were numerous in peritoneal exudate of rabbit that received an injection of egg albumen. They also were seen in the exudate of a rat after pyrrhol blue and in a human tubercular exudate.

Only an occasional transitional form could be detected in mesenteric lymph node of a rabbit after repeated injections of lithium carmine, and in nodes and spleen of rat after pyrrhol blue.

Lymphocytes did not change to histiocytes in lymphoid organs of rabbit injected with egg white or egg yolk.

It is concluded that the chief factor influencing the development of lymphocytes to histiocytes and macrophages lies in the ability of the tissues involved in an inflammatory reaction to furnish the required number of histiocytes from sources other than lymphocytes. In lymphoid organs lymphocytes are rarely converted to histiocytes because of the presence of reactive reticulum, rather than because the lymphocytes are too immature, or need to be conditioned by circulation in the blood.

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THE IN VITRO BEHAVIOR OF ERYTHROCYTES IN HUMAN CEREBROSPINAL FLUID

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IT HAS frequently been taught that the presence or absence of crenation of erythrocytes in cerebrospinal fluid is a significant point in the differential diagnosis between a subarachnoid or intraventricular hemorrhage and a traumatic spinal puncture. It is the purpose of this communication to show that the presence of crenated red blood cells in spinal fluid is, of itself, without such diagnostic significance and to present some observations on the behavior of human erythrocytes in cerebrospinal fluid.

METHODS

Sterile technique was used at all times. Eight to fifteen milliliters of cerebrospinal fluid were collected by the usual method from the lumbar spinal sacs of patients who had diseases not involving the central nervous system or the cerebrospinal fluid. In order to be assured that these spinal fluids were normal each specimen was subjected separately to quantitative analysis for protein; then the specimens were pooled and the sodium, potassium, and chloride content were determined. For obvious reasons those spinal fluids wherein the behavior of homologous red cells was to be observed were not pooled but were subjected separately and individually to each of the chemical analyses mentioned. In all instances these chemical values were normal. As soon as aliquots for the chemical determinations had been secured the specimens were stored in sterile air-tight containers at 1° to 2° C. until needed for further use.

Crenation, Red Cell, and "Ghost" Cell Counts.—Twenty cubic millimeters of capillary blood were gently pipetted into 10 ml. of cerebrospinal fluid. When pooled fluid was used, the examiner's blood was added; when a single fluid was used, the patient's blood was added. The cells were suspended by gently inverting the tube three times; then, with as little agitation as possible, 1 ml. of the suspension was pipetted into each of 9 sterile 10 × 75 mm. cuvettes. These rubber-stoppered cuvettes, with their contents, were maintained thereafter at 37.5° C. in a constant temperature water bath. Red cell counts were made immediately, and at 2, 4, 8, 12, 24, 36, 48, and 60 hours. Because it was found that the rate of destruction of the red cells was materially increased by the agitation necessary for proper cell suspension, only one cell count was made from each cuvette. To effect uniform cell suspension the cuvette was struck sharply 10 times with the finger, then cell counts were made immediately without diluting fluid in the usual manner, using an American Optical counting chamber. The presence or absence of crenation was noted in each cell counted, and the percentage of crenated cells was calculated. Cells were considered crenated if there was any irregularity at all of the cell border. "Ghost" cells were counted at the same time. It was necessary to focus up and down with the fine adjustment of the microscope in order to find many of the "ghost" cells.

Measurement of Xanthochromia.—A red cell suspension in cerebrospinal fluid was prepared as described previously, and, as shown in Table I, the following dilutions of this suspension were made in 10 × 75 mm. cuvettes, using pooled cerebrospinal fluid as the diluent.

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TABLE I

TUBE NUMBER	1	2	3	4	5
Red cell suspension (ml.)	1.0	0.5	0.1	0.05	0.025
Pooled cerebrospinal fluid (ml.)	1.0	0.5	0.9	0.95	0.975
Red cells per mm.^{3} (approximate)	10,000	5,000	1,000	500	250

Each cuvette was stoppered with a rubber stopper and gently inverted once; then all were maintained at 37.5° C. in a constant temperature water bath. The red cells settled to the bottom of the cuvettes and the optical density of the supernatant fluid was determined at 12-hour intervals throughout a 5-day period of time in a Coleman spectrophotometer at a wave length of 415 mμ. This setting was found to be the absorption maximum for the color produced and corresponds with the findings of Crosby and associates.[1]

Van den Bergh Test for Cerebrospinal Fluid.—The method as originally described by Leschke[2] was used.

RESULTS

Crenation of Red Cells.—Ninety-eight to 100 per cent of the cells were found to be crenated upon immediate examination of the fluid in three cases of traumatic lumbar puncture (red cell counts ranging from 15 to 14,250 per cubic millimeter), in two cases of subarachnoid hemorrhage (red cell counts of 70,000 and 110,000 per cubic millimeter), and in every instance where red cells were added to cerebrospinal fluid in vitro. As shown in Fig. 1, the percentage of crenated cells gradually decreased on incubation at 37.5° C. until only 50 to 75 per cent of cells were found to be crenated after 24 to 60 hours. At no time, under these conditions, were less than 50 per cent of the cells crenated. The morphology of the cells, however, changed markedly on incubation. Immediately after mixing blood and cerebrospinal fluid, all red cells were essentially identical in appearance, being of the same size and having the same degree of crenation. After incubation for 24 to 48 hours, the cells that remained showed marked anisocytosis and poikilocytosis and there was much debris present.

Red Cell Count.—During the first 12 hours of incubation there was a gradual decrease in the number of intact red cells remaining (Fig. 1). During the subsequent 24 to 36 hours of observation there was a rapid and precipitous decrease, so that at the end of 48 to 60 hours there were almost no cells left. The red cells decreased in number somewhat more rapidly when homologous cells and cerebrospinal fluid were used than when pooled fluid and the examiner's red blood cells were employed. It may well be, however, that this small difference is more apparent than real, because of the inaccuracies inherent in the usual method of red cell quantitation. At no time was there any suggestion of agglutination of cells in cerebrospinal fluid.

"Ghost" Cell Count.—"Ghost" cells, the envelopes of erythrocytes without cytoplasm, first made their appearance after 6 to 8 hours of incubation as shown in Figure 1. They increased in number at about the same rate that the intact cells decreased in number. It was never possible, however, to account fully for the total decrease in intact red cells by the increase in "ghost" cells. This may be due to the fact that "ghost" cells float at various levels in the counting

chamber; since it is necessary to focus up and down with the fine adjustment to find them, they possibly were not all seen.

Development of Xanthochromia.—In the in vitro studies xanthochromia was visible to the naked eye at about 4 hours after mixing the blood with spinal fluid when the cuvettes which contained 1,000 or more red blood cells per cubic milliliter of spinal fluid were compared with a tube of distilled water in good light. The development of color progressed slowly at first, but increased markedly after 12 to 24 hours' incubation and was complete at 48 to 60 hours as shown graphically in Fig. 1. The substance formed appeared to

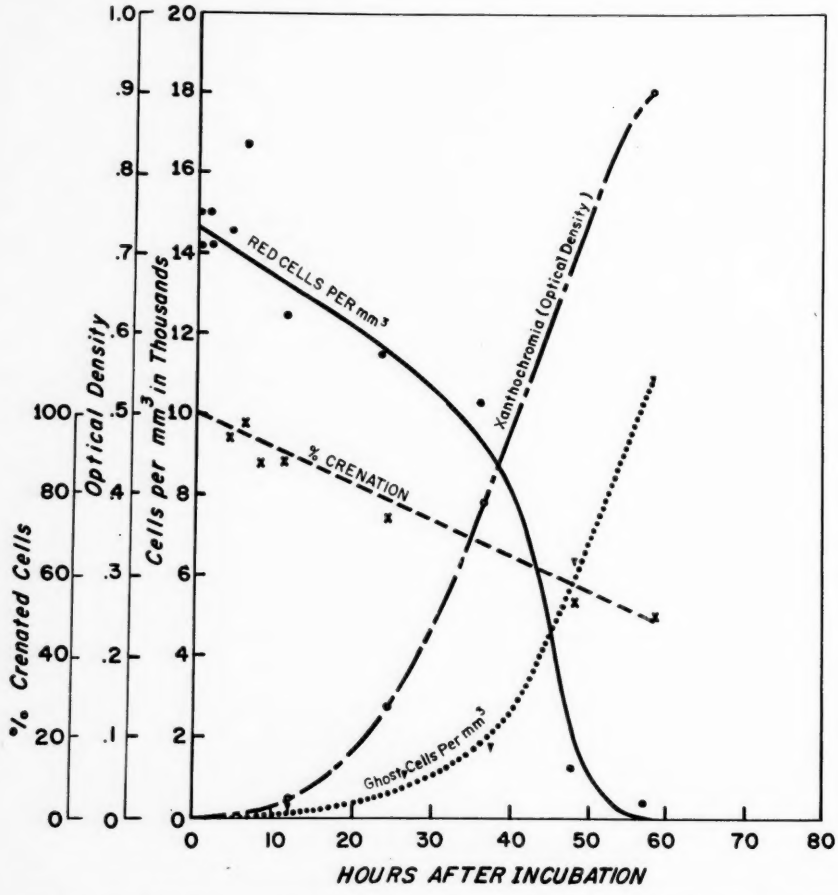

Fig. 1.—Changes in percentage of intact red cells showing crenation, red blood cell count, "ghost" cell count, and intensity of xanthochromia on incubation of human cerebrospinal fluid to which intact human erythrocytes were added in vitro.

be hemoglobin. The absorption spectrum curve of the fluid was very similar to that of a hemoglobin solution of approximately the same concentration prepared in sodium carbonate (Fig. 2). When the supernatant xanthochromic spinal fluid was mixed with an equal volume of 0.1 N HCl and heated for 30 minutes at 50° C., a substance was formed which had an absorption curve

identical to the absorption curve of acid hematin as given by Hawk and coworkers[3] (Fig. 3).

The absorption spectrum of the supernatant xanthochromic spinal fluid developing in vivo after subarachnoid hemorrhage was quite different from that of hemoglobin, however, as shown in Fig. 2. Acid hematin could not be derived from these fluids.

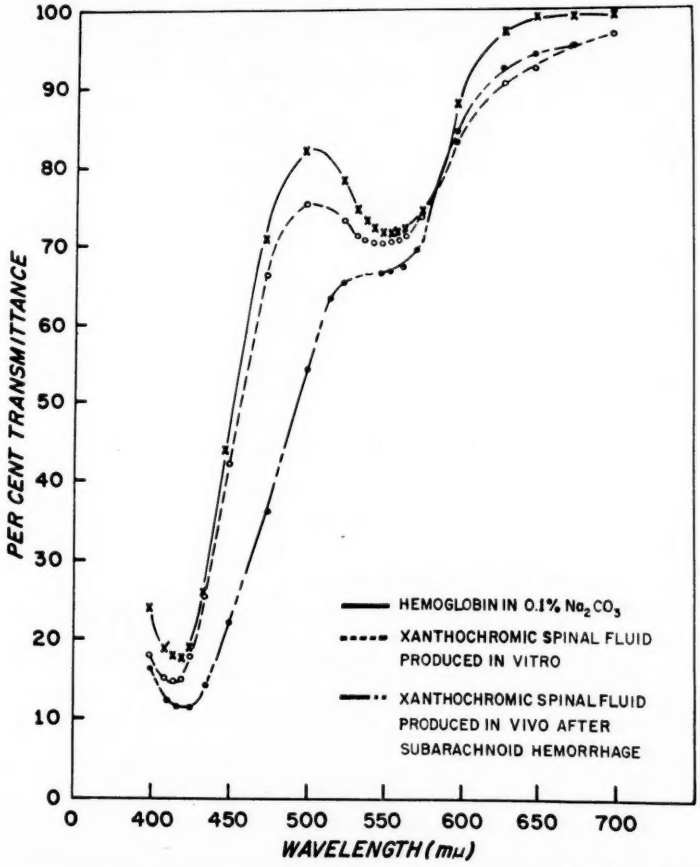
PER CENT TRANSMITTANCE
WAVELENGTH (mμ)
HEMOGLOBIN IN 0.1% Na_2CO_3
XANTHOCHROMIC SPINAL FLUID PRODUCED IN VITRO
XANTHOCHROMIC SPINAL FLUID PRODUCED IN VIVO AFTER SUBARACHNOID HEMORRHAGE

Fig. 2.—Absorption spectra of hemoglobin in 0.1 per cent Na_2CO_3, human spinal fluid after 60 hours of incubation at 37.5° C. with human erythrocytes, and xanthochromic spinal fluid from a case of subarachnoid hemorrhage. The intensities of color were similar at the point of maximum absorption.

The Van den Bergh reaction of cerebrospinal fluid incubated with or without added cells was consistently negative. This was true even of specimens incubated with added cells for as long as 60 hours. That a detectable degree of coloration was present in these latter specimens is evidenced by their having an optical density of 0.70 or more. In contradistinction to this the Van den Bergh reaction on specimens of grossly xanthochromic spinal fluid from two cases of subarachnoid hemorrhage was positive. Optical densities at 415 mμ on these specimens were as low as 0.13 and as high as 0.95, the quantitative bilirubin being 0.50 and 0.75 mg. per 100 ml., respectively.

DISCUSSION

Crenation of Red Cells.—The origin of the belief and teaching that the presence or absence of crenation of erythrocytes is significant in the differential diagnosis of bloody spinal fluid is obscure. The authors of several present-day textbooks[4-6] either stated or implied quite strongly that crenation is important, whereas other authors[7, 8] ignored the point completely. The idea possibly stems from the descriptions of bloody spinal fluid published in 1904 by Froin,[9] who listed, among other observations, the presence or absence

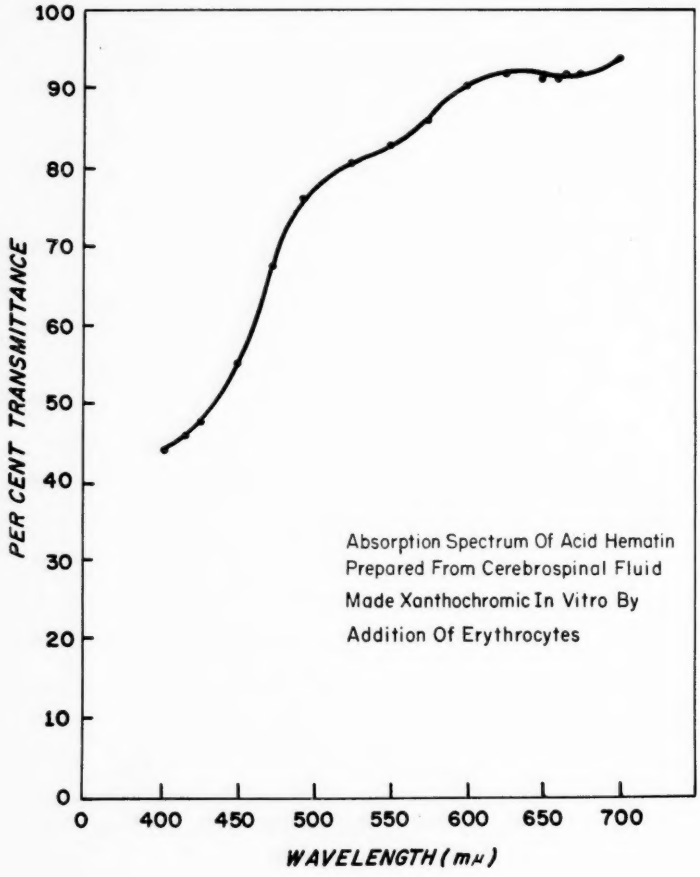

Fig. 3.

of crenation of red cells. Froin, however, did not list crenation of cells as one of the criteria of differentiation between a "bloody tap" and subarachnoid or intraventricular hemorrhage. It is possible that the idea rose in several places from the many readers of Froin's excellent articles. Symonds,[10] for example, gave the findings of two of Froin's cases of subarachnoid hemorrhage in which the morphologic appearance of the red cells is described as being normal until the seventh and tenth days, respectively, at which time "many crenated reds" were found. However, since cerebrospinal fluid is somewhat hypertonic,

containing 163 meq. per liter base as compared to plasma containing 155 meq. per liter base,[11] one would anticipate that crenation of erythrocytes would occur within a matter of seconds after admixture of blood and cerebrospinal fluid. This is what was observed here, the alteration occurring immediately. The presence of crenated red cells in spinal fluid, therefore, appears to be of no significance in the differential diagnosis between a traumatic spinal puncture and a subarachnoid or intraventricular hemorrhage.

Changes in Red Cell Count.—The rate of disappearance of erythrocytes from cerebrospinal fluid after their admixture in vivo has not been finally determined. De Jong[5] stated that in vivo the red cells have largely disappeared from the spinal fluid within two to three days after a subarachnoid hemorrhage. Biggart[7] stated that they begin to disappear after the first day. Simmonds,[12] in an effort to settle the matter, injected erythrocytes labeled with P^{32} into the subarachnoid spaces of rabbits and observed that 90 per cent of the cells had disappeared within 48 hours after injection. In his excellent study of the reactions of the meninges to blood, Hammes[13] stated that intact red cells could be seen in microscopic sections of meningeal tissue seven days after the original hemorrhage. He also stated that the first breakdown of blood cells was noted 16 to 32 hours after the onset of symptoms. He further noted that small recurrences of bleeding, especially in fatal cases, are to be expected. It may be, therefore, that cells found in the subarachnoid space at intervals longer than two days after the onset of symptoms are in reality due to recurrence of bleeding.

In vitro we found that cells almost completely disappeared after 60 hours of incubation. Admittedly, cells aggregated at the bottom of a cuvette in a water bath are not subjected to the same conditions as cells circulating freely in the subarachnoid space. Nevertheless, it seems likely that the time of disappearance of red cells in vitro corresponds fairly closely to that observed in vivo.

The actual route of disappearance of erythrocytes from the subarachnoid space has recently been questioned by Simmonds,[12] who presented evidence that they are reabsorbed into the circulating blood, probably via the subarachnoid villi. It is possible that the disappearance of at least some of the red cells from the spinal fluid may be explained on this basis. However, Leschke[2] conclusively demonstrated that bilirubin was at least partially responsible for the xanthochromia of cerebrospinal fluid subsequent to subarachnoid hemorrhage, thereby implying that destruction of red cells occurs in these instances. There is abundant other evidence that bilirubin is present in spinal fluid following subarachnoid or intraventricular hemorrhage.[9, 14] In a nonjaundiced individual the bilirubin in cerebrospinal fluid must come from hemolyzed red cells. In the in vitro studies described in this report there was observed good correlation between the disappearance of red cells and the increase in concentration of hemoglobin. In these studies the subarachnoid villi obviously were not present for reabsorption of the red cells; therefore, the sole mechanism for red cell disappearance under these in vitro conditions can only be one of red cell degeneration or hemolysis.

"Ghost" Cells.—As one of the confirmatory criteria for subarachnoid hemorrhage, Scully[15] included the presence of "ghost" cells. In one of our cases of subarachnoid hemorrhage, we also observed these cells in fairly large numbers. The formation of "ghost" cells in vitro begins six to eight hours after admixture of red cells and spinal fluid and progresses proportionately to the decrease in intact red blood cells. These red cell envelopes are rather easily recognized and, if seen in large numbers in any bloody spinal fluid which has not been vigorously agitated, probably indicate that the cells have been in contact with fluid for several hours. Therefore, the presence of "ghost" cells in bloody spinal fluid which has been promptly examined and not subjected to great agitation is strong evidence for the probability of subarachnoid or intraventricular bleeding rather than a traumatic spinal puncture.

Xanthochromia.—The rate of development of xanthochromia in vivo is fairly well established, color being present within four hours after the onset of symptoms.[4, 5, 16, 17] The degree of xanthochromia increases for a few days, and then, if there is no recurrence of hemorrhage, begins to decrease after the sixth to tenth day.[4, 17] These observations correspond well with our in vitro studies as to time of development of xanthochromia, in that color was visible to the naked eye in the supernatant fluid after four hours' incubation. Because the sterile fluids appeared cloudy after four days' incubation, they were discarded since this cloudiness precluded observations on the rate of disappearance of xanthochromia.

The xanthochromia observed in the present in vitro studies does not appear to be identical with that which occurs in vivo. The substance which develops in vitro has a rose-yellow color when fresh as compared with the canary-yellow color developing in vivo, and is very similar in appearance to the color of hemoglobin freshly prepared from whole blood in similar low concentrations. In addition the absorption spectra of hemoglobin and the xanthochromic spinal fluids prepared in vitro are very similar as compared to the dissimilar absorption spectra of the xanthochromic spinal fluids from patients with subarachnoid hemorrhage (Fig. 2). Acid hematin can be produced from the spinal fluids made xanthochromic in vitro, but not from those wherein xanthochromia develops in vivo. The Van den Bergh reaction was repeatedly negative in those fluids made xanthochromic in vitro, whereas the test was briskly positive on the xanthochromic spinal fluids of patients with subarachnoid hemorrhage. The evidence, therefore, is strong that the substance produced upon incubation of erythrocytes in cerebrospinal fluid in vitro is hemoglobin, and whatever substance is responsible for the transformation of hemoglobin to bilirubin in vivo is absent in vitro.

SUMMARY AND CONCLUSION

In vitro and in vivo observations were made on the rate and degree of red cell disappearance, development of crenation of red cells, "ghost" cells, and xanthochromia in spinal fluid. These studies showed that red cells in spinal

fluid become crenated immediately and disappear completely within 60 hours of contact with spinal fluid. "Ghost" cells develop within six to eight hours and xanthochromia is evident grossly within four hours after cells gain access to spinal fluid if the initial red cell concentration is 1,000 or more per c.mm. These findings were identical in both the in vitro and the in vivo studies. The nature of xanthochromia of bloody spinal fluid was studied and, in vitro, is due to hemoglobin, in contrast to bilirubin in vivo. The reason for this disparity was not discovered.

From these data the following conclusions were made: (1) Crenation of red blood cells in spinal fluid occurs immediately and is of no differential diagnostic significance between a traumatic spinal puncture and a subarachnoid or intraventricular hemorrhage. (2) Red blood cells in spinal fluid disappear within 60 hours in vitro and in vivo. (3) "Ghost" cells appear in bloody spinal fluid within six to eight hours after admixture of blood and spinal fluid. (4) Xanthochromia of bloody spinal fluid in vitro is due to hemoglobin in contrast to bilirubin in vivo. This color is apparent within four hours in the supernatant of bloody spinal fluid both in vitro and in vivo. (5) The presence of "ghost" cells and xanthochromia in spinal fluid is diagnostically indicative of previous recent subarachnoid or intraventricular hemorrhage provided the patient is not jaundiced and the spinal fluid is examined immediately.

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A STUDY OF CERTAIN ANTAGONISTIC ACTIONS OF PITUITARY GROWTH HORMONE AND CORTISONE

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With the Technical Assistance of P. G. Green

IN A series of sharply defined experiments, Kramár and co-workers[1-6] have shown that the hormones of the pituitary-adrenal cortical system exert a major controlling influence on the resistance of the cutaneous capillaries. These studies showed that both ACTH and cortisone elevate the value in normal dogs and albino rats while adrenalectomy causes a permanent decrease in both species. After adrenalectomy the capillary resistance is raised by cortisone but not by ACTH or desoxycorticosterone. In both species, under specified conditions, there is an inverse relationship between capillary resistance and the level of circulating eosinophils. In both dog and albino rat, nonspecific physical as well as emotional stress causes typical changes in capillary resistance. In these animals STH causes a lowering of the value below normal and is antagonistic to the effect of cortisone. On the basis of their studies, these authors believe that the actual level of capillary resistance is principally determined by the balance of these two hormones.

Wilhelmj and co-workers[7] used the value of the capillary resistance and the level of the circulating eosinophils as joint indices of the approximate degree of activity of the pituitary-adrenal cortical system.

It is generally recognized that many of the actions of pituitary growth hormone (STH) and cortisone are mutually antagonistic. The present studies were undertaken because it was believed that they might help in the understanding of certain changes that we have observed in blood pressure, heart rate, capillary resistance, and eosinophil counts during fasting and realimentation with luxus consumption diets high in carbohydrate, protein, or animal fats.[8, 9]

METHODS

Eight dogs, 4 male and 4 female, were used and no differences depending on sex were noted. Blood pressure was determined by the auscultatory method of Allen[10] with certain modifications.[11, 12] Capillary resistance was studied by the negative pressure method of Kramár and Simay-Kramár.[1] Eosinophil counts were made in a Spencer bright-line hemocytometer using the diluting fluid of Manners.[13]

The pituitary growth hormone used was Horner's Somatrofin Lot C35-39J. Schering's cortisone acetate was used throughout.

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DOSAGES AND METHODS OF ADMINISTRATION

1. *Pituitary Growth Hormone.*—Preliminary studies showed that 0.25 mg. per kilogram per day caused insignificant and uncertain changes in blood pressure and capillary resistance, whereas 1.0 mg. per kilogram per day caused a marked and well-sustained elevation of blood pressure and an equally pronounced fall in capillary resistance below the normal level. A dose of 0.5 mg. per kilogram per day caused an intermediate effect on both. On capillary resistance, 3.5 mg. per kilogram per day caused approximately the same decrease as 1.0 mg. per kilogram per day, but there was a greater delay in the return to normal when STH was discontinued. On the basis of these findings, the standard dose employed was 1 mg. per kilogram per day.

2. *Cortisone.*—By trial it was found that the effects of 1 mg. per kilogram per day of STH were almost exactly neutralized by 2.5 mg. per kilogram per day of cortisone. These will be referred to as the *standard doses.*

The total daily amount of STH was given intramuscularly in two equal doses at 8:30 A.M. and 3:30 P.M. The total daily dose of cortisone was given intramuscularly at noon.

The diabetogenic properties of Somatrofin Lot C35-39J were very low; daily blood sugar determinations showed no elevation even with doses as high as 8 mg. per kilogram per day.

Another lot of Somatrofin GEL-1 had very high diabetogenic properties, and the results with this will also be described.

Throughout the entire study the determination of the capillary resistance and the eosinophil count were done between 7:00 and 8:00 A.M. before the first injection of STH, and the daily allowance of food was given in one feeding at 3:00 P.M.

RESULTS

1. *Studies on Blood Pressure and Heart Rate (Fig. 1).*—As mentioned before, 0.25 mg. per kilogram per day of STH caused no definite effects. In Fig. 1, *A* it is seen that 0 5 mg. per kilogram per day gave a slight effect while 1 mg. per kilogram per day (Fig. 1, *B*) caused a definite elevation of systolic pressure and heart rate, but no change in diastolic pressure. In Fig. 1, *C* it is seen that 2.5 mg. per kilogram per day of cortisone was without effect when given alone, but when given simultaneously with 1 mg. per kilogram per day of STH (Fig. 1, *D*), it caused complete neutralization of the STH effect.

Fig. 1, *A* and *B* were done on the same dog at different times. In Fig. 1, *A* the animal was receiving the regular kennel diet of Nutrena, and the control blood pressure was 122/42. In Fig. 1, *B* the animal was receiving a diet high in carbohydrate (cracker meal) fed at the luxus consumption level (120 cal./M^2/hr./24 hr.) after a prolonged fast, and the control pressure was 145/38, but in spite of the initial high systolic pressure, STH caused a further rise. The initial 3-day decrease in systolic pressure was not seen on the usual kennel diet and may be due to the sudden shift in metabolism from carbohydrate to fat caused by STH.[14, 15]

In other experiments not shown in Fig. 1 it was demonstrated that if the standard dose of STH was administered alone for seven days, systolic pressure and heart rate were markedly elevated, but the simultaneous administration of the standard dose of cortisone for three days caused a rapid fall of both to normal.

2. *Studies on Capillary Resistance (Fig. 2).*—The standard dose of STH caused a prompt and highly significant fall in capillary resistance below the control level. This is shown in Experiments A and C, which were performed on the same dog at different times. In Experiment A the dog was receiving

a luxus consumption diet high in carbohydrate given after a prolonged fast, and the control level of the capillary resistance was 24 cm. Hg. In Experiment C the dog was receiving the standard kennel diet of Nutrena, and the control level of capillary resistance was 16 cm. Hg. This difference in capillary resistance due to diet confirms previous findings.[7]

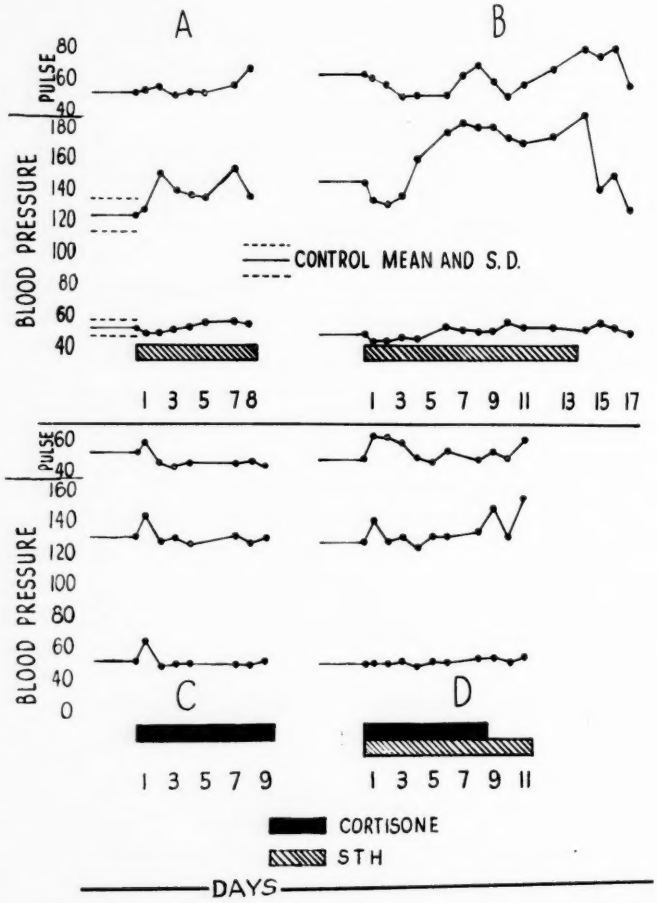

Fig. 1.—The effect of STH (1 mg./kg./day) and cortisone (2.5 mg./kg./day) on blood pressure and heart rate when given alone or simultaneously. *A*, The animal was receiving the standard kennel diet. *B*, The same animal was receiving a diet high in carbohydrate (cracker meal) administered after a preliminary fast.

The per cent fall in capillary resistance was greater on the high carbohydrate diet than on the kennel diet, and on the former diet the capillary resistance returned to the *normal* rather than to the high carbohydrate level after STH was discontinued. This may be due to the shift in metabolism from carbohydrate to fat caused by STH.[14, 15]

In Experiment B it is seen that the standard dose of cortisone given alone caused a prompt elevation of capillary resistance, while Experiments C and D show that when cortisone and STH were administered simultaneously, the usual effect of STH was neutralized.

In other experiments not shown in Fig. 2 it was found that 3.5 mg. STH per kilogram per day caused approximately the same fall in capillary resistance as the standard dose, but that the return to normal after stopping STH was greatly delayed.

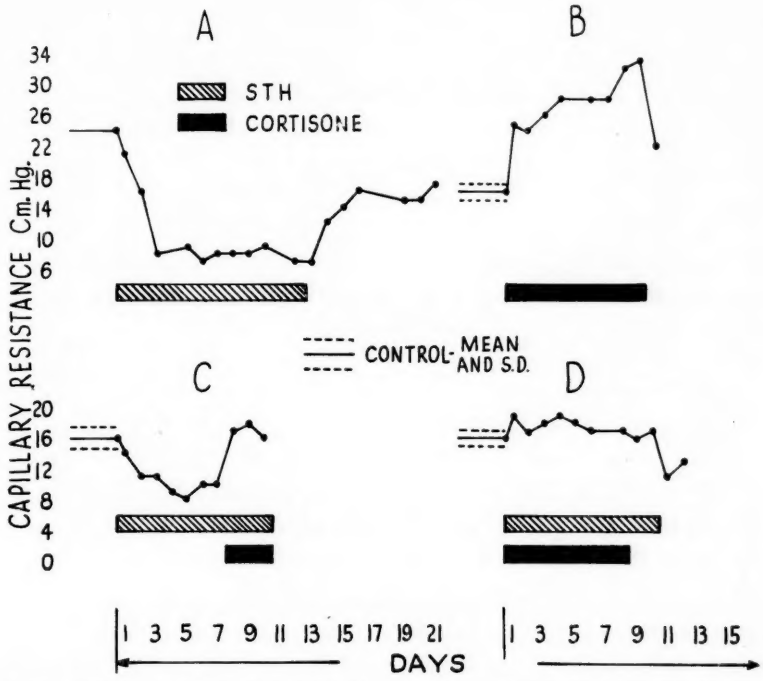

Fig. 2.—The effect of STH (1 mg./kg./day) and cortisone (2.5 mg./kg./day) on the resistance of the capillaries when given alone or simultaneously. *A*, The animal was on a high carbohydrate diet (cracker meal) administered after a preliminary fast.

3. *Studies on Fasting Animals.*—Fasting is an alarming stimulus which activates the pituitary-adrenal system and causes the release of adrenocortical steroids.[16] During prolonged fasting there is a progressive and marked rise in capillary resistance and a decrease in circulating eosinophils.[7, 17] If these effects are due to endogenous cortisone then they should be prevented or abolished by STH.

When healthy, well-nourished dogs are fasted, the capillary resistance usually reaches a maximal value of 70 cm. Hg (the highest vacuum used). The rise may be very rapid or it may be slow with occasional periods during which the values maintain a plateau for several days at levels considerably below the maximal value.[7] The plateaus below maximal values probably represent either an actual steady state of adrenocortical steroid liberation or simply a steady state of cortisone-like activity at submaximal levels. Guided by this concept, the effect of STH was studied during a plateau both at submaximal levels and at maximal levels of 70 cm. Hg.

Fig. 3 shows an experiment in which the capillary resistance rose with increasing rapidity until the twentieth day and then remained approximately

constant between 42 and 44 cm. Hg for five days. The injection of 1 mg. per kilogram per day of STH caused a prompt and marked fall, but within three days after stopping STH the value rose to 51 cm. Hg.

In Fig. 3 it is seen that the eosinophils had reached a very low level before STH was given, but they showed a progressive rise during the period of STH administration.

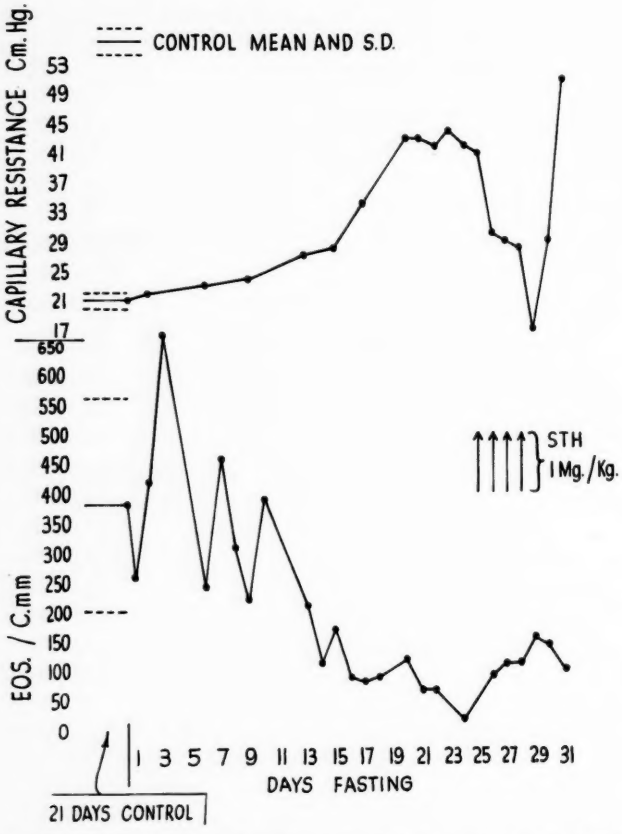

Fig. 3.—The effect of STH on the resistance of the capillaries and the eosinophil level in a fasting dog in which the capillary resistance was maintaining a plateau below the maximal value of 70 cm. Hg.

Fig. 4 shows an experiment in which capillary resistance was allowed to reach the maximal value of 70 cm. Hg before STH was given. First 2 and then 4 mg. per kilogram per day had only a transient, fleeting effect, and even 8 mg. per kilogram per day semed to have a somewhat wavering effect when compared with the steady depression caused by 1 mg. per kilogram per day in nonfasting dogs (Fig. 2). Within two days after stopping STH, capillary resistance returned to 70 cm. Hg.

The curve of body weight in Fig. 4 shows that weight loss amounted to only 0.1 kilogram during 5 days of STH administration, but after STH was discontinued, weight loss was accelerated and reached the anticipated level in three days.

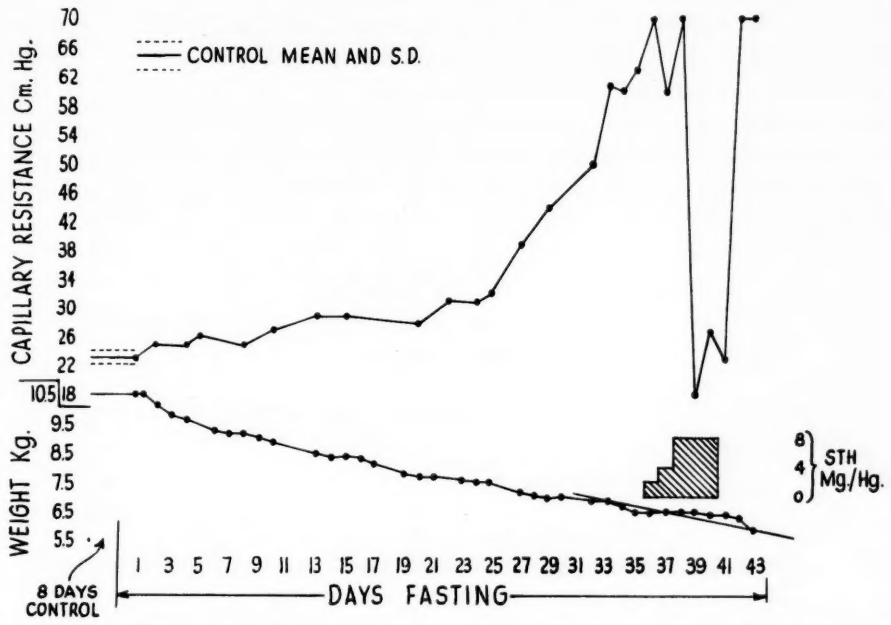

Fig. 4.—The effect of STH on the resistance of the capillaries and the body weight of a fasting dog in which the capillary resistance had reached the maximal value.

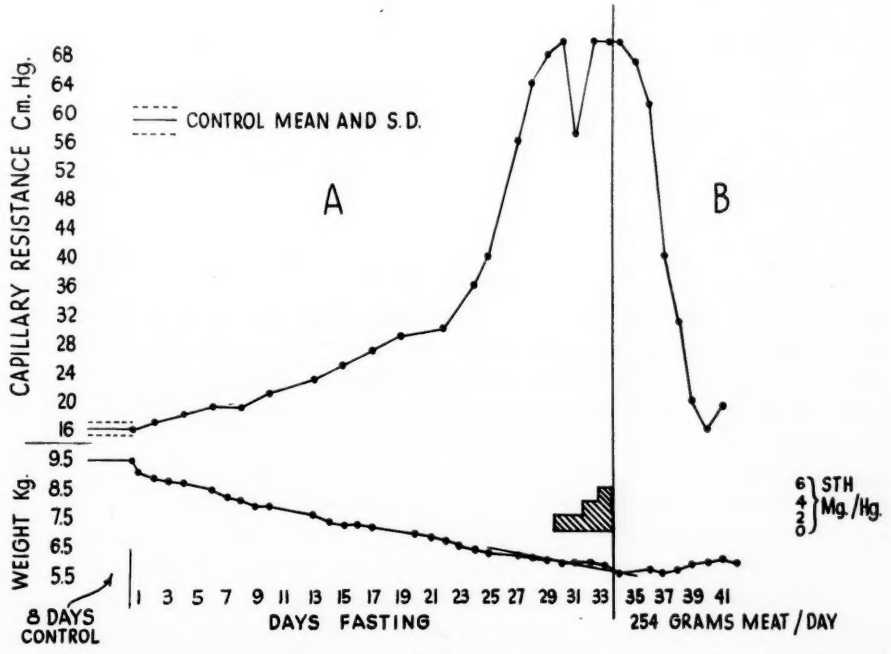

Fig. 5.—The effect of increasing doses of STH on the resistance of the capillaries and body weight in a dog in which capillary resistance had reached the maximal value. *B*, The effect of feeding 254 Gm. of horse meat daily.

Fig. 5 shows another experiment in which the capillary resistance reached the maximal value of 70 cm. Hg; 2, 4, and 6 mg. STH per kilogram per day were unable to neutralize the endogenous cortisone-like effect. Body weight maintained a plateau during STH administration, but dropped to the projected level the day after STH was discontinued.

When 254 Gm. of horse meat was fed daily, capillary resistance fell promptly and reached the control level in 7 days. This effect of meat confirms previous findings.[7]

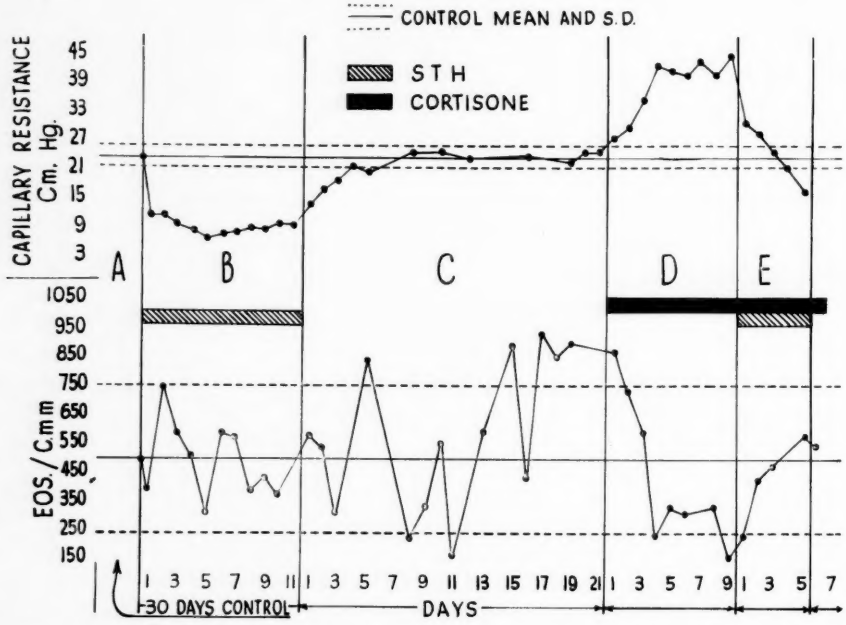

Fig. 6.—The effect of STH (1 mg./kg./day) and cortisone (2.5 mg./kg./day) on capillary resistance and eosinophil level of a normal dog when administered separately, *B* and *D*, and simultaneously, *E*.

4. *The Effect of Cortisone and STH on the Level of Circulating Eosinophils (Fig. 6).*—The behavior of the eosinophil count as illustrated in Fig. 3 suggests that when the eosinophil level is being depressed by endogenous cortisone, the administration of STH may cause an elevation. Does STH influence the eosinophil level directly or only by neutralizing the cortisone effect? The experiments illustrated in Fig. 6 were planned to answer this question. After a 30-day control period (6, *A*), the standard dose of STH was given for 11 days (6, *B*) and caused a marked and sustained decrease in capillary resistance but no change in the eosinopil level. During a 21-day recovery period (6, *C*) the capillary resistance returned promptly to normal while the eosinophil level continued to fluctuate within the control range. The standard dose of cortisone was then given alone for 9 days (6, *D*) and caused a prompt rise in capillary resistance and a fall in the eosinophil level. While cortisone was continued, the standard dose of STH was given simultaneously for 5 days (6, *E*). The STH promptly neutralized the cortisone effect, causing a rapid fall in capillary resistance and a rise in the level of the circulating eosinophils. From these experi-

ments it appears that STH has no direct effect on the eosinophil level and influences it only by neutralizing the effect of cortisone.

5. *Studies With an STH Preparation Having High Diabetogenic Properties.*—After completing these studies it was decided to repeat certain phases using a different STH preparation (Horner Lot GEL-1). In the first experiment this STH preparation neutralized the effect of cortisone on capillary resistance, but the decisive promptness previously noted was absent. In a second experiment, the effect of STH on the level of circulating eosinophils that was being depressed by cortisone was uncertain and doubtful. Other experiments showed the same lack of clear and decisive actions. After receiving this preparation (1 mg. per kilogram per day) for two days the animals showed typical symptoms of severe diabetes with blood sugar of 400 mg. per cent or more. Two animals died, and a histologic examination showed extensive destruction of the islands of Langerhans and intensely fatty livers. These observations suggest that the highly diabetogenic preparation was low in the factor responsible for the specific effects noted with the first preparation or that the animals were deteriorating so rapidly that normal responses were impossible.

DISCUSSION

It is not known whether there is a form of clinical or experimental hypertension due to endogenous STH, but if it does exist the blood pressure should be lowered by cortisone. It has been reported that adrenal cortical extract lowers blood pressure in some cases,[18] and it is interesting to speculate on the possible role of STH in these cases. Since large doses of exogenous cortisone also raise blood pressure, careful adjustment of the dose of cortisone would be necessary to secure exact neutralization of the STH effect without excess cortisone.

In the present experiments, it was shown that STH causes a high systolic pressure, a normal or low diastolic pressure, and an accelerated pulse rate. This is the type of cardiovascular response obtained when previously fasted animals are realimented with diets high in carbohydrate (rice or cracker meal) fed at the luxus consumption level, but the simultaneous changes in capillary resistance and eosinophil count suggest that STH is not the causative factor.[7]

Kramár[6] was the first to show that STH lowers capillary resistance below normal levels and that cortisone is antagonistic to this effect. The mechanisms involved in these changes are unknown, but since adrenal cortical hormones inhibit hyaluronidase activity,[19-22] the result of cortisone action could be a strengthening of the capillary wall with an increase in its resistance to rupture by external suction. Conversely the neutralization of cortisone action by STH might result in a weakening of the capillary wall and a lowering of its resistance to external suction.

The effect of STH on capillary resistance and the eosinophil count during fasting is additional evidence that the stress of fasting causes stimulation of the adrenal cortex with an increased liberation of adrenocortical steroids.

The present experiments are in agreement with the work of other investigators[23, 24] in suggesting that STH has no effect on the level of circulating eosinophils in normal dogs, but our studies also show that it may elevate the level when it is being depressed by cortisone. Since evidence is accumulating

that STH is liberated during stress, it may be necessary to consider this action when interpreting the stress response of the eosinophil level.

There is considerable indirect evidence suggesting that both cortisone and STH may be liberated in increased amounts during fasting, and it is obvious that both have actions which would be of benefit to the fasting animal.

In studies on prolonged fasting in which blood pressure, heart rate, eosinophil counts, capillary resistance, and body weight were determined daily,[7] it was observed that the behavior of these factors suggested a natural division of the fast into an early and a late stage. In the early stage blood pressure, heart rate, and eosinophil count fluctuate rather widely, but these fluctuations gradually become less, and in the late stage the blood pressure and eosinophil count decrease to low, stable fasting values while the capillary resistance reaches the maximal fasting height. From the findings reported in this paper the early stage of fasting may represent a period during which both STH and adrenocortical steroids are being liberated in such quantities that neither effect predominates and their antagonistic actions result in the wide fluctuations observed; the late stage of fasting suggests preponderance of a cortisone-like effect.

The rapid fall in capillary resistance caused by meat (Fig. 5) is also accompanied by a rise in eosinophils.[7] Unpublished studies have suggested that meat does not potentiate the action of STH since 0.25 mg. per kilogram per day, which was without effect on capillary resistance when the animal was receiving the kennel diet of Nutrena, was equally ineffectual on a high meat diet. The apparent inhibition of endogenous cortisone activity by meat may be due to the fact that when the metabolic mill of the fasting animal is flooded with protein, all of the endogenous cortisone-like steroids are immediately utilized in gluconeogenesis and none is available to influence capillaries or eosinophils, or it may be a mere cessation of the stress of fasting.

The neutralizing dose ratio of 2.5 mg. per kilogram of cortisone to 1 mg. per kilogram of STH will probably vary with different preparations, but more important is the variation in different species; Kramár,[6] using the same preparations in the albino rat, found the ratio to be 1 mg. per 100 grams body weight of cortisone to 2 mg. per 100 grams body weight of STH.

SUMMARY

With the preparations of STH and cortisone used in the present studies on dogs, it was found that 1 mg. per kilogram per day of STH and 2.5 mg. per kilogram per day of cortisone were almost exactly mutually antagonistic. These are referred to as the standard doses. The standard dose of STH caused a marked elevation of systolic blood pressure and pulse rate, which was antagonized by the standard dose of cortisone, which was without effect when given alone. On capillary resistance that standard dose of STH caused a prompt and significant fall, while the standard dose of cortisone caused a rise. When administered simultaneously, mutual neutralization of these effects occurred. STH has no direct effect on the level of circulating eosinophils, but causes an elevation when the eosinophil count is being depressed by cortisone. These findings suggest that the eosinophil level is not being depressed by endogenous cortisone in the normal nonstressed animal. During the late stage of prolonged fasting

there is a decrease in eosinophils and a rise in capillary resistance. These effects appear to be due to endogenous cortisone because both are reversed by STH in sufficient doses. When STH is administered to fasting animals it causes a prolonged plateau in the curve of body weight.

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CONTINUOUS RAPID INFRARED CO_2 ANALYSIS

Fractional Sampling and Accuracy in Determining Alveolar CO_2

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Hondo, Calif.

IN A steady respiratory state and at a constant atmospheric pressure and metabolic rate, the concentration of alveolar CO_2 is inversely proportional to alveolar ventilation. Hyperventilation causes a lowering and hypoventilation causes a rise of the alveolar CO_2 concentration; these changes are quantitatively proportional to the degree of ventilatory abnormality. Changes in metabolic rate will produce proportionate changes in alveolar CO_2 concentration unless a compensatory change in alveolar ventilation occurs.* In the normal state, alveolar CO_2 levels are maintained within rather narrow limits[2] independent of the metabolic rate or the size of the physiologic dead space. Thus, alveolar CO_2 concentrations can serve as a valuable guide for proper maintenance of CO_2 homeostasis during prolonged periods of artificial respiration required in poliomyelitis, anesthesia, or other conditions associated with altered breathing.

Various physiologic studies[3-8] have demonstrated that a rapid infrared CO_2 analyzer can be used for alveolar CO_2 determinations. Experience has shown, however, that careful attention to certain details is essential to make the procedure useful clinically and to avoid misleading or inaccurate data. This paper reports in detail the fractional sampling techniques which have been used in clinical determinations of alveolar CO_2 during a 17-month period. It presents data to establish the accuracy of these techniques and to compare them with existing methods.

Four different sampling techniques can be utilized. These can best be explained and differentiated by Fig. 1. The major points of distinction are

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*These statements can be verified by examination of the alveolar equation (1):

$$\dot{V}_A = \frac{R\dot{V}_{O_2}\ (P_B - 47)}{P_{A_{CO_2}}}$$

Where: $\dot{V}_A$ = Alveolar ventilation
R = Respiratory quotient
$\dot{V}_{O_2}$ = Rate of oxygen consumption
P_B = Barometric pressure
$P_{A_{CO_2}}$ = Alveolar CO_2 tension

This formula is derived from simple arithmetic equivalents.

as follows: First, in the two "total" sampling methods all of the air is passed through the analyzer, whereas in the two "fractional" sampling methods only a fraction of the air is passed through the analyzer. Second, the two "to-and-fro" sampling methods analyze both inspired and expired air, whereas the two "expiratory" sampling methods analyze only the expired air.

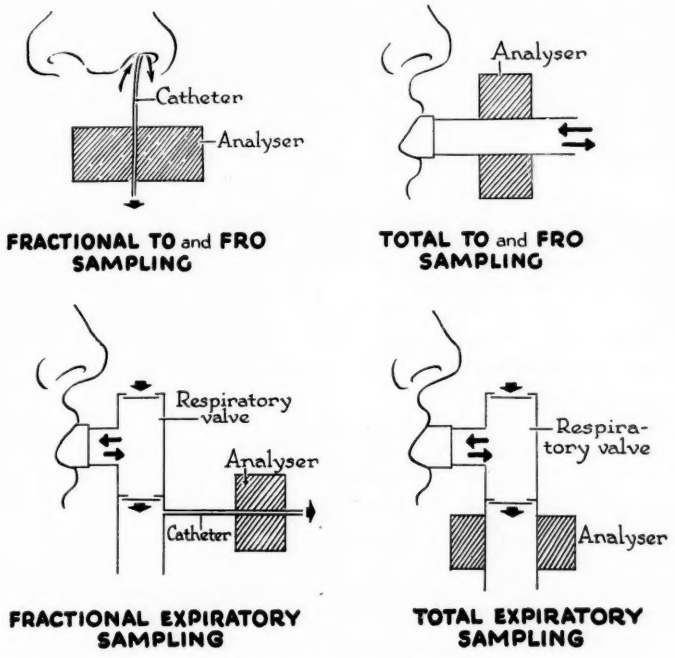

Fig. 1.—Sampling methods of infrared alveolar CO_2 analysis.

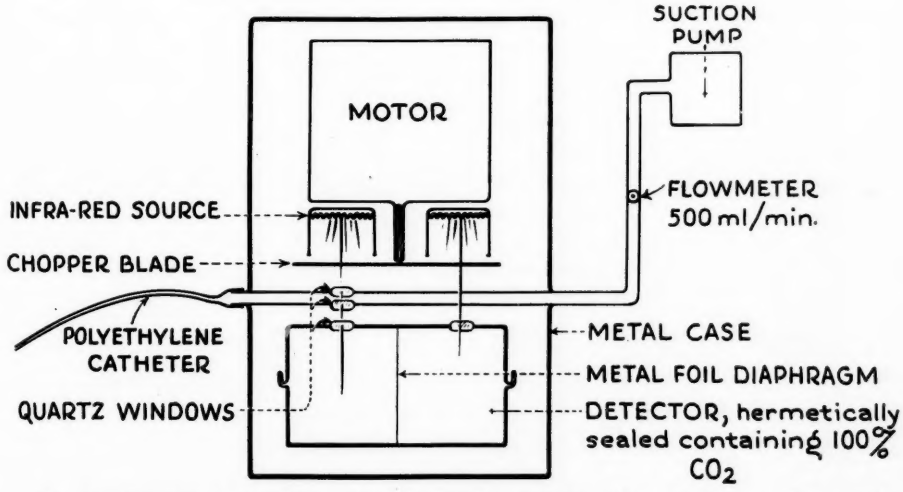

Fig. 2.—Fractional sampling with the rapid infrared CO_2 analyzer. The sample is pumped from the airway through the catheter and analyzer. The CO_2 in each chamber of the detector absorbs specific portions of the infrared spectrum, heating each chamber equally if no CO_2 is present in the sample. When CO_2 is present in the sample, the chambers are heated unequally, causing displacement of the diaphragm. The degree of displacement is detected electrically and recorded.

In the fractional to-and-fro sampling technique a very small catheter which does not interfere with breathing is placed in the nostril or open tracheotomy tube and a continuous sample is pumped through it into the infrared analyzer (Fig. 2). Thus, both inspired and expired air are sampled and analyzed. No mask, mouthpiece, or nose clip is used. This technique approaches an ideal method for investigating respiration, in that it does not interfere with the function which it purports to measure. Other sampling methods utilizing the passage of the total respired air sample through the rapid infrared CO_2 analyzer have been discussed by Elam, Brown, and Ten Pas.[9] In these methods the analyzer is located near the patient's mouth (to-and-fro sampling) or just beyond an expiratory valve (expiratory sampling).

MATERIALS AND METHODS

The instrument used in these studies was a Liston-Becker* Model 16 infrared absorption carbon dioxide analyzer equipped with a special cell for fractional sampling. An explanation of the operation of this instrument has been published.[10] The Liston-Becker analyzer differs from other commercially available analyzers in that its response to changes in carbon dioxide concentration is more rapid. Such rapid response is essential for following respiratory CO_2 changes.

In order to record these rapid changes, a specially damped, ink-writing Esterline-Angus milliammeter† or the DC amplifier of a Sanborn‡ direct-writing oscillograph recorder may be used. Either recording system is satisfactory for fractional sampling but, because of its slow response, the microammeter on the Liston-Becker amplifier case is suitable for the analysis of static or homogeneous samples only.

For routine clinical use it is desirable to make the instrument readily portable and simple to operate. These aims have been achieved by placing the analyzer and allied equipment on a small rolling table (Fig. 3). In the figure, the detector unit is to be seen in the upper left-hand corner. It is suspended by shock absorbers to minimize vibration and is oriented with the diaphragm of the detector cell in a vertical plane. Vertical orientation of the diaphragm tends to minimize the effects of inertia and shock. The amplifier and recorder rest on the table top, which is also mounted on shock absorbers. Shelves below the table top are fitted to hold six high pressure cylinders containing various mixtures of CO_2 in air for calibration. These tanks are connected to a common manifold which conducts the mixtures to a small plastic "artificial nostril" located on the side of the detector unit. The lower left-hand corner of the figure shows the pump and flowmeter. The pump is a Neptune Dyna-Flow§ diaphragm type equipped with needle valves for controlling rate of flow and a small chamber for smoothing the flow. Flow rates are measured with a Size C Rotameter.‖

The catheter itself deserves some discussion. It is a 9-inch length of polyethylene tubing of outside diameter 1.27 mm. and inside diameter 0.86 mm.¶ connected to a shortened 20 gauge needle. The catheter must be of such small size as to obtain dependable results. During preliminary studies a 5-inch length of 10 French rubber catheter was used, but the results were erratic and undependable. Further investigation of the characteristics of this sampling line was accomplished by means of a ⅛ square inch pneumotachograph made of 400-mesh wire cloth.[11] This study revealed that air flow in the sampling line was actually reversed during inspiration, because pressure changes in the nostril exceeded the pressure drop through the instrument. Such reversal of flow lengthened

*Liston-Becker Instrument Co., Inc., 649 Hope St., Springdale, Conn.

†Model AW DC milliammeter Range 0 to 5 Ma. with special measuring element to give 0.2 second response time, Esterline-Angus Co., Inc., P.O. Box 596, Indianapolis 6, Ind.

‡Sanborn Co., 39 Osborn St., Cambridge 39, Mass.

§No. 84404, Standard Scientific Supply Corp., 34 W. 4th Street, New York 12, N. Y.

‖No. 20735, Central Scientific Co., 1700 Irving Park Road, Chicago 13, Ill.

¶PE 90, Clay-Adams Co., Inc., 141 E. 25th Street, New York 10, N. Y.

the response time so much that measurements with the instrument were undependable. The problem was ultimately solved by using the small diameter polyethylene catheter, which produced a pressure drop of 40 mm. Hg across the catheter and sampling cell with I.D. 3.30 mm. tubing, at the usual flow rate of 500 ml. per minute. The small catheter not only insures continuous sample flow, but also minimizes the dead space from catheter tip to absorption window. Both factors tend to reduce response time.

Method for Clinical Sampling.—The instrument is unstable for many hours after it is turned on. To maintain optimum electrical stability, the instrument is disconnected only while it is being moved to the bedside. After being moved it is allowed to warm up for about 10 minutes. The only detrimental effect which has been observed from such continuous operation is the failure of the synchronous chopper motor in the detector unit after six to nine months of service.

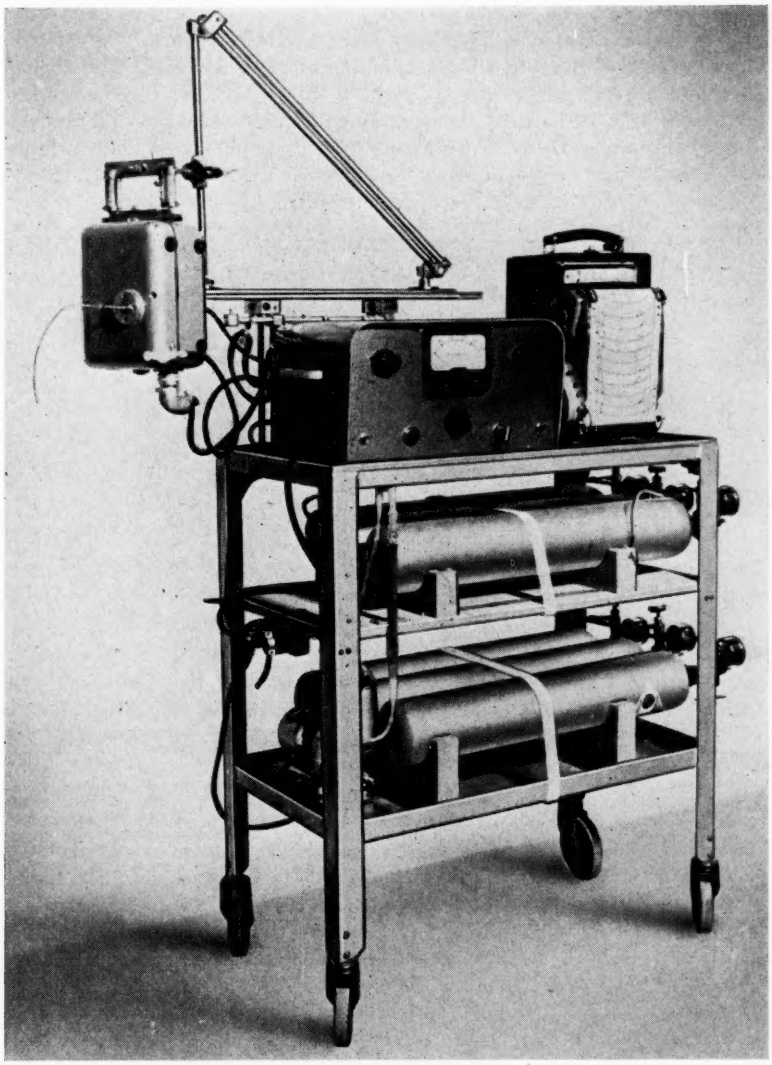

Fig. 3.—Rapid infrared CO_2 analyzer arranged as a portable unit. Upper left, catheter and pick-up unit; table top, amplifier and recorder; lower left, pump and flowmeter; lower, high pressure tanks for calibration mixtures.

The flow rate through the catheter tip is routinely set at 500 ml. per minute by means of the flowmeter. This rate seems to be a satisfactory compromise consistent with accurate sampling and rapid response. It is far less than the usual expiratory flow rates and volumes. Higher sampling rates reduce the instrument response time, but may exceed the expiratory flow rate. Lower rates increase response time and serve no useful purpose.

The free end of the polyethylene catheter is placed about 1 cm. into the nostril or open tracheotomy tube and is kept free of the sides of the nostril to avoid drawing secretions into the analyzer.

All routine determinations are made by means of a continuous recording, using the Esterline-Angus recording milliammeter. During inspiration only room air is sampled, which provides a continuous check on the stability of the instrument. The general patterns of CO_2 expiration curves while breathing room air have been described.[3, 9] The first phase of the expiratory CO_2 curve consists of a flat portion containing no CO_2. The second phase is an S-shaped curve of a rapidly rising CO_2 concentration. The third phase consists of a sloping plateau. The first two phases represent the washout of the dead space, whereas the last phase represents relatively pure alveolar gas. A typical recording is shown in Fig. 4. The record is examined immediately for the presence of a sloping alveolar plateau. If the expiratory curves are only sharp spikes and the alveolar plateau is not present, due usually to a small tidal volume and/or a rapid rate, the determination is not valid and the record is discarded. Even a single breath of sufficient volume to produce an alveolar plateau will provide a "spot check" of alveolar CO_2 concentration.

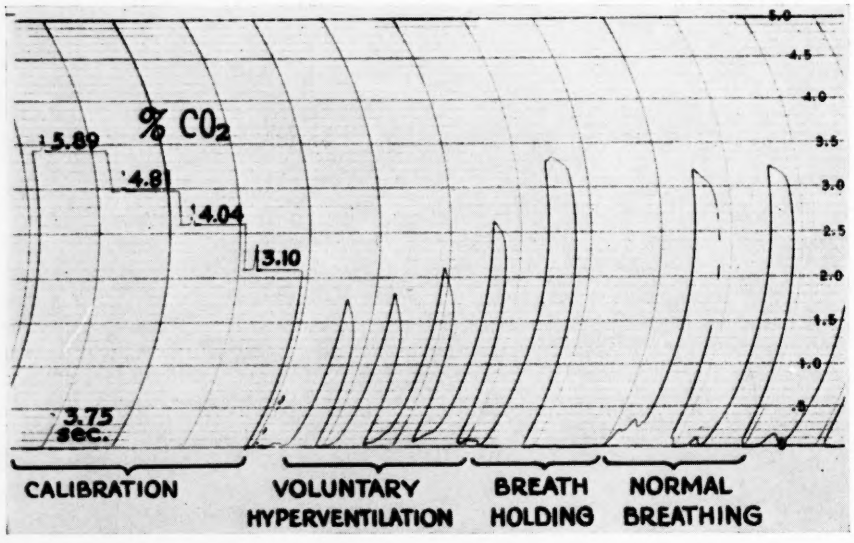

Fig. 4.—Continuous CO_2 record. Read from right to left. Note that each expiratory curve shows a sloping alveolar plateau.

Calibration of the instrument is easily accomplished by inserting the catheter tip into the plastic "artificial nostril" on the side of the detector, flushing the "nostril" with CO_2 mixtures of the appropriate concentrations, and making short recordings of each concentration. Calibration with six mixtures can be achieved in less than 30 sec. For routine use, calibration with not less than three mixtures approximately 1 per cent CO_2 apart and spanning the desired range of measurement is recommended. Due to the high flow resistance of the catheter, the absorption chamber of the sample cell is slightly below atmospheric pressure. Therefore, it is necessary that the catheter size and the sampling flow rate be the same at time of calibration as during a measurement. The instrumental sensitivity is slightly reduced with lowered pressures because fewer molecules of CO_2 are present in the sample cell.

Since the duration of most of our routine measurements is only a few minutes, calibration is accomplished at the end of each run to insure maximum accuracy. Calibration, of course, can be repeated as often as desired, but if the instrument is kept connected continuously as recommended, and its position is not changed, calibration is probably not necessary more often than once each hour. Frequent checks of the calibration over numerous four-hour periods have shown changes which would introduce a relative error of 4 per cent or less over the range of 0 to 6 per cent CO_2. If ambient temperature fluctuates widely between calibrations, greater errors may be introduced, because the sensitivity of the instrument increases with lower ambient temperature.

The calibrating mixtures are purchased in large cylinders and analyzed in duplicate by the Scholander technique.[12] The mixtures are then transfilled as required into the small cylinders which are mounted on the table. The small cylinders are evacuated prior to transfilling when the mixture being added contains a different percentage of CO_2 than that which was formerly in the cylinder. This transfilling procedure saves considerable analytic effort. Repeat analyses have been found to check within 0.02 per cent CO_2 even after several months. Mixtures of CO_2 in air are preferable to CO_2 in oxygen, because the background gases more nearly approximate the concentrations observed in most respiratory mixtures. Stow[13] has described this possible error and we have found that a relative error of about 7 per cent is introduced if mixtures in O_2 are used rather than mixtures in air.

The calculation of the results of a determination is relatively easy to do. The carbon dioxide concentrations of the calibrating mixtures are plotted against the respective values for response appearing on the recording. Cartesian, semilogarithmic, or logarithmic coordinates may be used for this purpose. Each detector cell has a characteristic response curve, and appropriate coordinates should be chosen to make the straightest calibration line. The peaks of the alveolar slopes recorded during the determination are arbitrarily chosen for measurement. One breath or an average of any number of peaks may be chosen for computation. The value for alveolar CO_2 is read from the calibration graph and reported as per cent CO_2 (dry).* It is also calculated and reported as tension (partial pressure) using the following formula:

$$P_{ACO_2} = F_{ACO_2} (P_B - 47)$$

P_{ACO_2} = Alveolar CO_2 tension in mm. Hg
F_{ACO_2} = Fraction of alveolar CO_2 (% ÷ 100)
P_B = Ambient barometric pressure
47 = Tension of water vapor in mm. Hg at 37° C.

If it is desired to use an expanded scale with a suppressed zero such as was used in the experiment shown in Fig. 5, this can be accomplished by adjusting a mechanical shutter on the reference side of the detector system so that zero response is obtained when a gas of the desired CO_2 concentration is present in the sample cell.

Method for Establishing Accuracy.—The accuracy of the described method was checked by comparison of alveolar CO_2 tensions obtained with the infrared analyzer with arterial CO_2 tensions in blood samples obtained simultaneously. The subjects were either patients with respiratory paralysis due to poliomyelitis or normal subjects. An indwelling needle

*The analysis is performed on moist gas but the calibrations are made with dry gas. The analyzer used in these studies has sufficient interference from water vapor so that we have chosen to use the water vapor correction factor (P_B – 47) rather than P_B in calculating the tension. Our data in support of this statement are as follows: (P_B = 759) a mixture consisting of 4.07 per cent CO_2 in air (dry) was bubbled slowly through water at 37.0° C. for twenty minutes. Readings were then taken with the infrared analyzer on the flowing moist gas and on the same gas dried with anhydrous calcium sulfate. Three sets of readings were made alternately with identical results within the reading error. The results were:

	Milliamperes output	% CO_2
Dry	3.40	4.07
Wet	3.37	4.04

Our interpretation was that this test revealed an interference from water vapor which nearly balanced the dilution by water vapor. The expected dilution effect alone would have given a reading of $4.07 \times \frac{712}{759} = 3.82\%$.

Elam[14] stated that analyzers may differ in their response to water vapor so that the analyzer in use should be tested in order to obtain the highest accuracy.

was placed in the femoral or brachial artery after preliminary procaine anesthesia. After 20 minutes a 10 ml. blood sample was withdrawn anaerobically over a one-minute period using a lightly oiled syringe containing heparin and sodium fluoride. During the blood withdrawal infrared analysis was performed in the manner described previously. The infrared alveolar CO_2 tension was calculated independently. The blood was analyzed in duplicate for CO_2 content by the method of Van Slyke and Neill. The pH was determined at 37° C. by a Beckman pH meter with an anaerobic glass electrode. The hematocrit was determined by the method of Wintrobe. From these data the arterial CO_2 tension was computed using the nomogram of Singer and Hastings.[15]

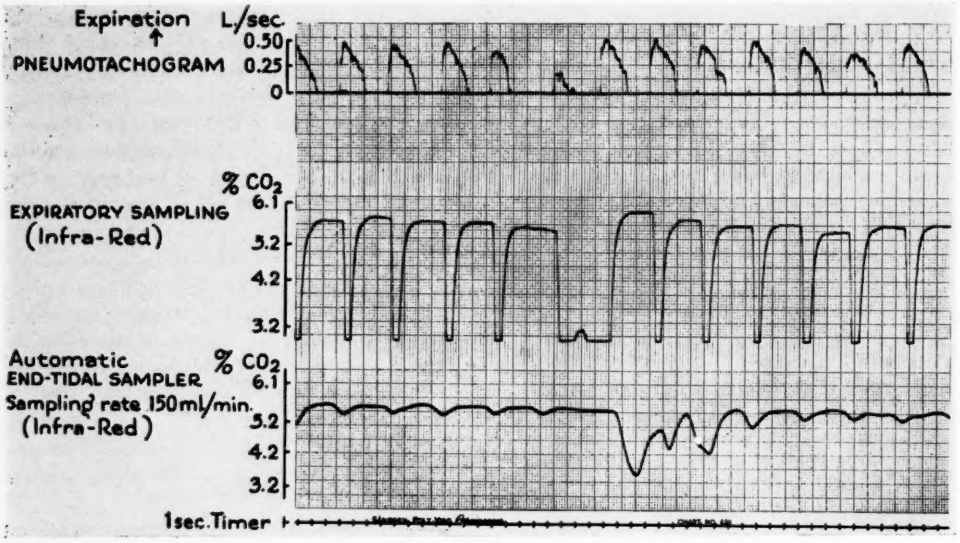

Fig. 5.—The effect of irregular breathing on alveolar CO_2 analysis. Read from left to right. The CO_2 scales have a suppressed zero. The small breath causes inadequate washout of the dead space. This is detected by both sampling methods only by a continuous recording.

Method for Determining Response Time.—The over-all response time of the analytic system can best be examined by presenting the catheter tip with a square wave of a fixed concentration of CO_2. This was accomplished by placing a toy balloon in a large glass jar and inflating the balloon with a CO_2 mixture until it nearly filled the large jar. The catheter was placed in the jar so that it could sample air from around the balloon until the balloon was exploded. When the balloon was ruptured, its contents were released within the jar almost instantaneously. The instant of the explosion was marked by means of a sensitive mechanoelectric pressure transducer with small dead space connected to a Sanborn multichannel recorder. The infrared CO_2 was recorded on an adjacent channel at a paper speed of 50 mm. per second.

Method for Comparing With End-tidal Sampler.[16]—This comparison of fractional infrared alveolar CO_2 analysis and the end-tidal sampler was made because the end-tidal sampler has often been used in physiologic studies for the collection of gas for the determination of alveolar gas concentrations. The end-tidal sampler* used had a dead space of 30 ml. The end-tidal sample was drawn at a rate of 150 ml. per minute through an infrared analyzer and recorded on one channel of a Sanborn Polyviso. A second infrared analyzer was arranged to sample at a rate of 500 ml. per minute from a point just beyond the expiratory valve (fractional expiratory sampling). Thus, in effect, both methods were used to sample from the same point. A pneumotachograph[11] on the expiratory line was used to record expiratory flow rates.

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RESULTS

Accuracy.—Determination of arterial CO_2 tension is the best available approximation of mean or "effective" alveolar CO_2 tension[17, 18] The mean alveolar CO_2 tension is a better index of the level of ventilation than a single instantaneous value. Therefore, the arterial tensions were used to evaluate the infrared method of alveolar CO_2 analysis. Table I shows the comparison of arterial values with infrared determinations using two sampling techniques. No experiments have been omitted. The fractional to-and-fro sampling method shows a mean deviation of –0.9 mm. Hg from the arterial tension with a standard deviation of the mean of ± 1.8 mm. Hg. These values are shown graphically in Fig. 6. The comparison of infrared total expiratory sampling with arterial CO_2 tensions shows poorer correlation than the fractional to-and-fro sampling, probably because of the inherently greater apparatus dead space. The dead space of the breathing valve was only 30 ml., but the dead space up to the sample cell of the analyzer was an additional 67 ml. Expiratory sampling is not to be recommended as a routine method as most breaths will show a perfectly flat plateau even if the breath is too small in volume to present alveolar air to the analyzer. With other sampling methods, the presence of a sloping alveolar plateau validates the sample.

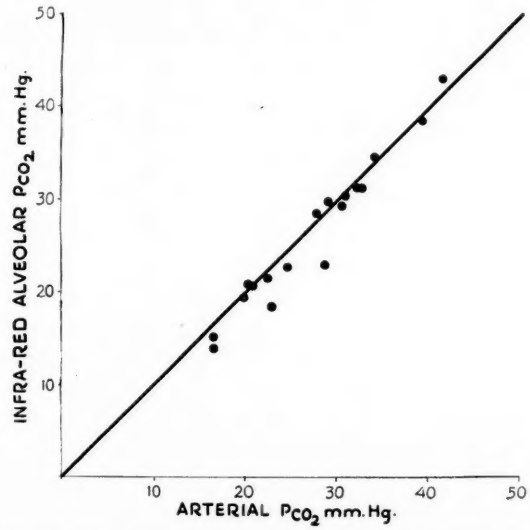

Fig. 6.—Accuracy of fractional to-and-fro sampling technique in determining alveolar CO_2. The arterial CO_2 tension is the best available approximation of mean or "effective" alveolar CO_2 tension. The points represent experimental values. The line represents all the points having equal arterial and alveolar tensions.

Response Time.—The mean time lag from balloon explosion to initial analyzer response was 0.15 sec. The mean additional time for 90 per cent of total response was 0.63 sec., giving a total delay of 0.78 sec. for 90 per cent response. These figures were obtained when using the recommended polyethylene catheter and sampling rate. The volume of the catheter was 0.15 ml. The volume of the sampling cell up to the quartz window was 0.80 ml., giving a total dead space of 0.95 ml. Most of the data reported here was ob-

TABLE I. COMPARISON OF ARTERIAL CO_2 TENSION WITH INFRARED ALVEOLAR CO_2 TENSION

NO.	AMBIENT BAROMETRIC PRESSURE (MM. HG)	ARTERIAL CO_2 CONTENT (MEQ./L.)	PH	HEMATOCRIT (%)	CALCULATED ARTERIAL CO_2 TENSION (MM. HG)	INFRARED ALVEOLAR CO_2 TENSION (MM. HG)	CO_2 TENSION DIFFERENCE (IR-ARTERIAL) (MM. HG)
Fractional To-and-Fro Sampling							
1	757	13.8	7.33	33	28.7	23.1	-5.6
2	760	12.0	7.55	44	16.5	15.0	-1.5
3	756	21.1	7.42	48	40.0	43.2	+3.2
4	756	15.8	7.60	48	20.4	20.7	+0.3
5	758	19.0	7.53	43	27.8	28.5	+0.7
6	756	12.8	7.47	41	20.6	20.8	-0.2
7	756	14.6	7.45	24	22.5	21.4	-1.1
8	758	19.7	7.49	39	30.5	29.3	-1.2
9	758	17.5	7.40	30	30.8	30.4	-0.4
10	757	15.3	7.47	39	24.6	22.7	-1.9
11	754	14.3	7.36	42	29.0	29.8	+0.8
12	755	19.1	7.43	35	32.7	31.3	-1.4
13	757	12.9	7.57	35	16.5	13.9	-2.4
14	758	15.1	7.48	29	22.8	18.3	-4.5
15	756	20.8	7.46	42	34.0	34.7	+0.7
16	761	21.0	7.41	45	39.2	38.6	-0.6
17	759	15.9	7.59	38	19.8	19.3	-0.5
18	756	18.5	7.43	40	32.2	31.4	-0.8
19	760	11.9	7.44	36	19.8	18.4	-1.4
20	761	12.5	7.52	35	17.8	17.1	-0.7
21	761	20.9	7.50	44	32.6	31.0	-1.6
22	765	17.9	7.48	43	28.8	29.8	+1.0
Mean							-0.9
Standard deviation of the mean							±1.8
Total Expiratory Sampling							
1	764	20.9	7.43	44	36.8	35.0	-1.8
2	764	20.7	7.44	44	36.0	37.0	+1.0
3	754	14.6	7.36	42	30.0	29.6	-0.4
4	754	13.5	7.42	42	24.6	21.4	-3.2
5	755	19.3	7.42	35	33.8	33.8	0.0
6	755	18.4	7.47	35	29.1	26.8	-2.3
7	757	13.6	7.50	35	20.0	16.7	-3.3
8	757	11.9	7.62	35	13.7	11.0	-2.7
9	758	14.0	7.53	29	18.8	15.6	-3.2
10	758	13.4	7.60	29	16.0	11.1	-4.9
11	758	19.3	7.37	47	40.0	31.8	-8.2
12	756	21.6	7.41	42	39.0	35.2	-3.8
13	756	21.7	7.40	42	40.0	31.5	-8.5
14	761	21.7	7.41	45	40.5	38.7	-1.8
15	761	21.5	7.41	45	40.3	34.7	-5.6
16	759	16.8	7.51	38	25.0	22.3	-2.7
17	756	19.1	7.38	40	37.0	32.6	-4.4
18	760	10.7	7.54	36	14.4	12.5	-1.9
19	760	10.4	7.57	36	13.3	11.0	-2.3
Mean							-3.2
Standard deviation of the mean							±2.4

tained using this larger sampling cell. The manufacturer has recently designed a sampling cell using tubing of 0.8 mm. I.D. This new cell has a volume of only 0.14 ml. up to the window, giving a total dead space from catheter tip to window of only 0.29 ml. This new cell showed a mean lag from balloon explosion to initial analyzer response of 0.06 sec. with a mean additional time of 0.21 sec. for 90 per cent of total response, giving a total time delay of 0.27 sec. for 90 per cent response. These response times are considerably longer

than those reported for total sampling methods.[9] However, many patients cannot tolerate the added dead space and interference with breathing imposed by total sampling methods. Here, fractional sampling is the only method able to obtain alveolar CO_2 values.

The pressure drop across the new cell and catheter is 74 mm. Hg at a flow rate of 500 ml. per minute. The instrument was calibrated at various sampling rates and hence at various sample pressures. The results are shown in Table II. From these data, it can be estimated that changes in sampling rate of ± 50 ml. per minute will introduce an absolute error of approximately ± 0.1 per cent CO_2 in the normal physiologic range.

TABLE II. CHANGE IN CALIBRATION WITH CHANGES IN SAMPLING RATE

SAMPLING RATE (L/MIN.)	PRESSURE DROP ACROSS CATHETER AND CELL 0.8 MM. I.D. TUBING (MM. HG)	INSTRUMENTAL RESPONSE TO 5.89% CO_2 GAIN 4-5 (MILLIAMPERES)
0.0	0	4.00
0.2	31	3.88
0.3	48	3.80
0.4	62	3.73
0.5	74	3.66
0.6	98	3.57
0.7	124	3.46

End-tidal Sampler.—Results using the automatic end-tidal sampler were compared with the infrared determination of alveolar CO_2 in normal subjects, in patients with poliomyelitis and on a mechanical lung which had a constant output of CO_2 into the "alveolar space." The end-tidal sampler gave samples which correlated well with the infrared alveolar CO_2 determinations in all adult cases having constant tidal volumes greater than 350 to 400 ml. At smaller tidal volumes there is contamination of the end-tidal sample with dead space air.

It is interesting to note that one patient exhibited cyclic breathing. At the lowest tidal volumes, there was inadequate washout of the dead space, as shown by the to-and-fro infrared recordings. These low points, of course, could be discarded on visual inspection of the tracings. Using the adequate points of the to-and-fro infrared curves for computation, results showing a deviation of + 1.0 mm. Hg from arterial CO_2 tension were obtained, whereas the end-tidal sample was in error by –5.8 mm. Hg. The effect of only one small breath on the end-tidal sample is shown in Fig. 5. It must be noted that the end-tidal sampler responds slowly and erratically to these inadequate breaths. Pneumotachograms recorded from patients with poliomyelitis receiving artificial respiration show intermittent glottic closure, usually associated with swallowing. When this occurs, the patient may miss the major portion of one inspiration, and the small breath thus produced will contaminate an end-tidal sample with dead space air. The continuous recording of the infrared analyzer immediately detects the dead air space contamination and obviates this difficulty, which would be encountered with the end-tidal sampler.

DISCUSSION

Alveolar CO_2 concentration is not a fixed entity, but varies both with "time" and "space." It varies with "time," because the concentration of alveolar CO_2 is dependent upon the particular phase of inspiration or expiration. The concentration is reduced during inspiration and increased during expiration because the relatively constant output of CO_2 is released into a lung space which is changing in size with the respiratory cycle. This variation in concentration has been proved mathematically[19, 20] and has been demonstrated during expiration by serial sampling methods[21, 22] and by rapid analytic techniques.[3, 23] Space variation means that variation present in the concentration of CO_2 in different portions of the lung at any given time.[24, 25]

With these facts, it is easily seen that no method can do more than approximate the mean or "effective" alveolar CO_2 concentration. The estimation of arterial P_{CO_2} is undoubtedly the best approximation of mean alveolar P_{CO_2}, but it is both time-consuming and difficult. Moreover, blood analyses, by either direct or indirect methods, have inherent errors which make it impossible to determine gas tensions with an accuracy of better than $\pm$ 2 mm. Hg. The infrared alveolar CO_2 analyses reported here show variations which are somewhat greater than this, but they are very adequate for clinical use. The speed and ease with which infrared determinations may be made largely compensate for the inherent errors of the method. Also, the new sampling cell with minimal dead space is expected to reduce the errors of the infrared method.

The infrared techniques probably cannot be applied successfully in any condition, such as emphysema, in which there are marked variations in ventilation-perfusion ratios in various parts of the lung. However, the infrared analyzer can be used to detect such variations,[26] so that errors due to this abnormality can be avoided.

It is self-evident that in order to analyze alveolar air, alveolar air must be presented to the analyzer. In other words, the expiratory volume must be large enough to sweep all dead space air out of the airways. This volume of air is considerably larger than the physiologic dead space and has been termed the "kinetic dead space." It is impossible to define limitations of the analyzer in so far as respiratory rates and volumes are concerned. Experiments with a mechanical lung indicate that breathing rates up to 40 per minute may be followed adequately, providing the tidal volumes are sufficiently large. We have made many satisfactory determinations on 2-year-old children. It is fortunate that the presence of an alveolar plateau on the tracings can give assurance that alveolar air is being sampled. It is not possible to estimate alveolar CO_2 levels on the basis of sharp peaks seen on the records.

Older methods of alveolar air sampling and analysis have defects not present in the infrared method. The Haldane-Priestley method requires excellent training in order to deliver good samples, and because of the increase of alveolar CO_2 concentration with time, does not give extremely accurate samples. Further, only a spot check is possible. The automatic end-tidal sampler has been shown to be defective in cases of small breaths or irregular

breathing. The fractional sampling method of continuous infrared alveolar CO_2 analysis is superior to these older methods, in that it has the ability to give rapid breath-to-breath analyses; it does not interfere with breathing; it does not require a trained subject; the results may be checked immediately by inspection of the records.

The method has been used extensively in the wards and has proved to be a valuable and practical clinical guide for providing adequate artificial ventilation for patients with respiratory paralysis due to poliomyelitis. Since the alveolar CO_2 tensions of normal individuals breathing air at sea level in the supine position are maintained at a mean value of 40 mm. Hg,[2] we have arbitrarily used a range of $\pm$ 5 mm. Hg as representing normal conditions. Thus, alveolar CO_2 tensions above 45 mm. Hg are indicative of hypoventilation, and tensions below 35 mm. Hg are termed "hyperventilation." Hypoventilation is desirable to the body economy only in case of metabolic alkalosis; and conversely, hyperventilation is advantageous only in case of metabolic acidosis or at high altitudes. Severe degrees of either hypoventilation or hyperventilation are undesirable. The use of the infrared CO_2 analyzer provides a simple, rapid guide for proper regulation of respirator pressure and rate.

Prior to the use of the infrared CO_2 analyzer, the chief guide for proper ventilation of the respirator patient was the measurement of tidal and minute volumes.[27] However, this method requires an educated guess as to the amount of ventilation any given patient requires. Experience has indicated that once patients are overventilated, they tend to demand continued overventilation, thus tending to perpetuate a vicious cycle.

Various indirect measurements of CO_2 tension have been proposed recently. One is based on electroencephalographic changes.[28] Another test depends on the respiratory efforts by the anesthetized patient.[29] A third is a photocolorimetric method,[30] and another is based on the measurement of oxygen consumption and ventilation volumes.[31] The advantages of the direct and rapid estimation of alveolar CO_2 tension by the infrared technique are obvious.

It has been possible, by strict attention to details, to remove the infrared CO_2 analyzer from its role as a laboratory curiosity and to place it in the wards as a valuable clinical tool for the guidance of proper artificial ventilation. Technicians have been trained in the use of the analyzer and in the calculation of results. A total of 1,416 determinations have been performed during 17 months of use at this hospital and 2,759 determinations have been done at the Communicable Diseases Unit of the Los Angeles County Hospital during nine months of use. Attention to detail is, however, mandatory. A recent paper[32] describing the use of the infrared analyzer during anesthesia reported data of such nature as to preclude any practical use. The cause of these apparent errors is not obvious from the material presented.

Further use of these methods should be of value in the study and diagnosis of heart disease, lung disease, and the hyperventilation syndrome. The value of the CO_2 analyzer in anesthesia has been discussed in a series of papers by Elam and associates.[9, 33, 34]

SUMMARY

Detailed techniques are presented for a method of alveolar CO_2 determination which utilizes a fractional to-and-fro continuous sampling method and a rapid infrared absorption gas analyzer with a direct recording system. The validity of the method was established by comparing the alveolar CO_2 values with arterial CO_2 tensions of blood drawn simultaneously. Twenty-two such comparisons reveal a mean deviation of –0.9 mm. Hg with a standard deviation of the mean of ± 1.8 mm. Hg.

The limitations of the method are discussed. The method is compared with other methods for determining alveolar CO_2 concentration. The practical value of the infrared method as a clinical guide to proper administration of artificial ventilation is demonstrated.

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THE EMERGENCE OF ANTIBIOTIC-RESISTANT GRAM-NEGATIVE BACILLI

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INTRODUCTION

THE recent widespread use of antimicrobial agents in clinical medicine has changed the pattern of bacterial infections in man. Changes which have suppressed acute infections have been of therapeutic value, others have introduced new problems in medicine. The present report gives evidence that the number of drug-resistant strains of bacteria causing urinary tract infections has significantly increased. Since pyelonephritis is found in 15 per cent of post-mortem examinations[1] and urinary tract infections are among the most common recurrent infectious diseases for which antimicrobial therapy has been effective, this is a challenging finding.

METHODS AND MATERIALS

"Preantibiotic" Strains.—This group of organisms consisted of species of gram-negative bacilli commonly associated with urinary tract infections. Specimens were obtained from the American Type Culture Collection, where they had been maintained in a lyophilized state. All specimens had been lyophilized from a calcium carbonate-skim milk medium. Except for one specimen (*Klebsiella pneumoniae* 10273, Julliane Type C), which had been collected in 1948, all strains had been incorporated into the collection no later than 1946, while 22 had been incorporated prior to 1944.

"Recent" Strains.—Similar strains of gram-negative organisms isolated from the urine of persons with urinary tract infections between August, 1953, and February, 1954, were studied. All strains were considered to play an etiological role in these infections. The criteria for significance were clinical evidence of infection together with the presence of 1,000 or more organisms per milliliter of urine as determined by a quantitative culture method.[2] Many of the patients had received courses of one or more antibiotics before the urine was cultured.

In Vitro Sensitivity Tests.—The method of Braude and Dockrill[3] was modified for the purpose of this study as follows: Twofold dilutions of antibiotic in 4 ml. of tryptose-phosphate broth (Difco) were prepared with final concentrations from 2 to 32 μg per milliliter. In some instances wider ranges of concentrations were tested. Tubes were inoculated with 0.1 ml. of a broth culture (10^9 organisms) in the logarithmic growth phase (5 to 7 hours) and incubated at 37° C. for 18 hours. The lowest antibiotic concentration causing macroscopic inhibition of growth was reported as the "sensitivity level." This reading approximated absolute bacteriostasis. The level of partial inhibition was 1 to 5 tubes lower. Bacteria

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not inhibited by 32 μg per milliliter were considered resistant. One person did the sensitivity studies using the same lots of each antibiotic in all studies.

Lyophilization.—Thirteen recent strains were cultured for 18 hours in tryptic-digest broth, then centrifuged at 1,420 × G (2,500 r.p.m.) for 15 minutes. After decantation, 5.0 ml. of calcium carbonate-skim milk medium* was added to the sediment and the mixture lyophilized for 24 hours. Cultures were put through this procedure four times and the sensitivity levels compared with those found before lyophilization. Bacterial virulence was also compared in these lyophilized and original cultures.

Bacterial Virulence.—Eighteen-hour tryptose-phosphate broth cultures were washed twice by centrifugation (1,420 × G for 5 minutes) and resuspension in 0.85 per cent saline solution (pH 6.95). The bacterial suspensions were diluted with saline solution to a density of 40 to 70 units (Klett-Summerson colorimeter without a filter). From this saline suspension, 1.0 ml. was injected intraperitoneally into albino mice weighing 19 to 22 grams. Control mice were given intraperitoneal injections of broth or saline solution. Each bacterial strain was inoculated into groups, which usually contained six or more mice. Pour-plate counts gave values of $10^{10} \pm 10^{2}$ viable bacteria per milliliter. Only deaths occurring within four days were included; survivors were listed with mean survival times of 96 hours for purposes of calculation.

Statistical Evaluation.—Differences noted in various comparisons were evaluated by preparing fourfold tables and applying χ^2 tests. Yates correction for discontinuity in small series was also applied in each instance. Probabilities of less than 2 per cent (0.02) were considered significant.

RESULTS

Observations on Shifting Bacterial Sensitivity to Antibiotics.—The number of recent strains sensitive to a given concentration of antibiotic is given in Table I. With the method used, a sensitivity level of 32 μg per milliliter was the highest value (chloramphenicol and the tetracycline analogues) at which a clinical response to the usual therapeutic dosage (1 to 2 Gm. a day) was uniformly observed. The total number of bacterial strains sensitive to any one of these antibiotics was quite small, ranging from 16 per cent sensitive to streptomycin to 55 per cent sensitive to chloramphenicol. Among the preantibiotic strains the proportion "sensitive" ranged from 70.5 per cent sensitive to streptomycin to 92.5 per cent sensitive to oxytetracycline. The data in Tables I and II indicate that the incidence of bacterial strains resistant to any given antibiotic is quite variable from one species to another.

Few strains of recently isolated *Aerobacter aerogenes* and Proteus sp. were sensitive to streptomycin, chlortetracycline, or oxytetracycline. The number of preantibiotic and recent strains of these organisms sensitive to chloramphenicol was not significantly changed.

Strains of recently isolated *Escherichia coli* were often sensitive to the broad-spectrum antibiotics, as were the strains of this species isolated before the antibiotic era. It should be noted that most of the strains of *E. coli* included in our preantibiotic group were resistant to streptomycin. Although most of the recently isolated strains of *E. coli* were sensitive to the broad-spectrum agents, there was a trend toward increasing resistance.

Strains of *Pseudomonas aeruginosa* in the recent series were markedly resistant to streptomycin, whereas most strains in the preantibiotic series were sensitive. These findings were in contrast to the low incidence of resistance

*Skim milk powder (Difco) 60 Gm., $CaCO_3$ 6.0 Gm., H_2O 300 ml.

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TABLE I. IN VITRO SENSITIVITIES OF "RECENT" STRAINS

BACTERIAL SPECIES	NUMBER OF STRAINS	NUMBER OF STRAINS INHIBITED BY 32 μG/ML. OR LESS			
		STREPTOMYCIN	CHLORAMPHENICOL	CHLORTETRACYCLINE	OXYTETRACYCLINE
Aerobacter aerogenes	77	6	43	28	26
Proteus species	55	6	19	11	7
Escherichia coli	45	10	38	34	31
Pseudomonas aeruginosa	24	3	5	13	15
Misc. gram-neg. "coliforms"	49	15	33	33	29
Total	250	40	138	129	108
% inhibited		16	55	52.5	43

TABLE II. IN VITRO SENSITIVITIES OF "PREANTIBIOTIC" STRAINS

BACTERIAL SPECIES	NUMBER OF STRAINS	NUMBER OF STRAINS INHIBITED BY 32 μG/ML. OR LESS			
		STREPTOMYCIN	CHLORAMPHENICOL	CHLORTETRACYCLINE	OXYTETRACYCLINE
Aerobacter aerogenes	5	4	3	5	5
Proteus species	5	3	4	4	3
Escherichia coli	4	1	4	4	4
Pseudomonas aeruginosa	5	4	1	4	5
Misc. gram-neg. "coliforms"	8	7	7	7	8
Total	27	19	19	24	25
% inhibited		70.5	70.5	89	92.5

found in both the recent and older strains to chlortetracycline and oxytetracycline. Both preantibiotic and recent strains of this organism were uniformly resistant to chloramphenicol.

The miscellaneous category of organisms in the preantibiotic group consisted of the following: *K. pneumoniae,* 3 strains; *A. cloacae,* 2 strains; *E. freundii,* 2 strains; and *E. intermedium,* 1 strain. In the recent group this category consisted of similar species and in addition included strains of paracolon and *Alcaligenes fecalis.* In this category there was the same over-all trend toward increased numbers of resistant strains, particularly to streptomycin. Again there were general species variations in the preponderance of resistant strains; however, the number of strains of any one species was too small to permit individual comparisons.

Observations on the Effect of Preservation on Bacterial Cultures.—Comparison of these two groups of bacteria was valid only if preservation had not altered those characteristics which were to be compared. The colony morphology of the species in each group was similar and was not altered by repetitive lyophilization. As a check on the sensitivity method and on the stability of unlyophilized cultures stored at 5° C., the sensitivity levels of the preantibiotic organisms and several of the recent strains were repeated after a two- to three-month interval. Repeat sensitivity values did not vary by more than two tubes, which was within the error of the method.[4]

Since the preantibiotic organisms had been preserved by lyophilization, thirteen representative strains from the recent group were lyophilized four times. This was at least once more than the maximum number of times which any of the preantibiotic strains were processed. These procedures did not alter the in vitro sensitivities of the organisms.

The virulence of the eleven strains of *Ps. aeruginosa, E. coli, A. aerogenes,* and Proteus sp. from the preantibiotic group was compared with thirteen strains of the same species from the recent group. In the preantibiotic group the mortality was 19 animals out of 60 at 96 hours, with a mean survival time of 71.6 hours. The mortality for the thirteen recent strains was 38 animals out of 82, with a mean survival time of 58.5 hours. These differences were not significant. Neither were consistent differences in virulence found in simultaneous studies carried out with the nonlyophilized and the four-times-lyophilized variants.

Correlation Between Resistance and Antibiotic Administration.—The recent group of organisms were isolated almost entirely from patients with histories of previous antibiotic therapy. However, at least thirteen of the infections studied occurred in persons who had definitely not received antibiotics during the preceding year or at any time for a urinary tract infection. The in vitro resistance of these thirteen strains was similar to that of the remainder of the group.

DISCUSSION

Evidence is presented in this study that a greater proportion of the bacterial strains associated with urinary tract infections is becoming resistant to antibiotics. There is precedent for this phenomenon in other types of human infections. Well over one-half of the strains of staphylococci isolated from clinical infections are resistant to penicillin.[5-7] This is in contrast to the existence of almost uniform sensitivity to penicillin among strains of staphylococci when this drug was first introduced. It is also in contrast to the sensitivity of staphylococci isolated from infections occurring in parts of the world where this antibiotic has had little usage.[8] A similar appearance of resistant tubercle bacilli is now in process. Patients who continue to shed tubercle bacilli are likely to acquire strains resistant to either streptomycin, para-amino salicylic acid, or isonicotinic acid hydrazide, depending upon the type of treatment used.

Comparison of the in vitro sensitivities of coliform bacilli, *Bacillus proteus,* and *Pseudomonas aeruginosa,* reported by Frank, Wilcox, and Finland in 1950,[9, 10] with recent results from our laboratory as well as those reported by Kirby and associates,[11] lead us to feel that an important increase in the resistance of these species to the broad-spectrum antibiotics had occurred. Further evidence that a significant shift in drug resistance is taking place has been supplied by Schneierson,[12] who found that strains of *E. coli* often are now resistant to chlortetracycline.

The present study was based on a comparison of the antibiotic sensitivities of a group of organisms recently recovered from urinary tract infections with a group of these same species isolated before the antibiotic era. No evidence could be found that the techniques used in storing the control cultures altered their susceptibility to antibiotics. The advantages of being able simultaneously to compare the antibiotic susceptibility of two groups of bacteria rather than merely to compare previously recorded results seem to outweigh the disadvantages of this approach.

The degree of change in bacterial sensitivity noted in this study varied for the antibiotic and for the particular species tested. Even individual strains within a single species were not uniform in their response. The general pattern of these changes is illustrated in Table III. Perhaps the one generalization which can be drawn from this information is that there is now in progress an emergence of antibiotic-resistant bacteria in urinary tract infections, the full extent of which remains to be determined. It is obvious that rational therapy of urinary tract infections must be based in part on in vitro sensitivity studies on the causative organism.

TABLE III. DEVELOPMENT OF ANTIBIOTIC RESISTANCE DURING ANTIBIOTIC ERA

BACTERIAL SPECIES	ANTIBIOTICS STUDIED			
	STREPTOMYCIN	CHLORAMPHENICOL	CHLORTETRACYCLINE	OXYTETRACYCLINE
Aerobacter aerogenes	+	0	+	+
Proteus species	+	0	+	+
Escherichia coli	R	0	0	0
Pseudomonas aeruginosa	+	R	0	0
Misc. gram-neg. "coliforms"	+	0	0	±
Total	+	0	+	+

+ Significant increase ($p \leq 0.02$) in number of resistant strains.
± Probably significant increase ($p \leq 0.10$) in number of resistant strains.
0 No increase ($p \geq 0.10$) in number of resistant strains.
R Most of "preantibiotic" strains resistant.

The clinical importance of this increase in antibiotic resistance in genitourinary infections is augmented by the fact that a high percentage of individuals with such infections have an obstruction to urine flow as a primary factor in the pathogenesis of their disease. Unless these mechanical factors are eliminated or alleviated, successful antibacterial therapy can be bacteriostatic in nature only, and, of necessity, must be repeated frequently or continued for extended periods of time. This situation is highly conducive to the emergence of bacterial strains which are resistant to the antimicrobial agents which are used.

SUMMARY

A significant decrease is found in the total number of strains of gram-negative bacilli sensitive to streptomycin, chlortetracycline, and oxytetracycline when a group of 27 strains of gram-negative bacilli isolated prior to 1946 are compared with a group of 250 similar strains isolated from individuals with genitourinary infections between August, 1953, and February, 1954. The magnitude of the change is variable from one species to another, being most marked among the strains of *Aerobacter aerogenes* and the Proteus species.

In the same comparison there has been no significant change in the number of strains sensitive to chloramphenicol, although there has been a trend toward increased resistance.

The decrease in proportion of sensitive strains is not associated with changes in colony morphology or with a loss of virulence for mice.

Repeated lyophilization does not alter the levels of antibiotic susceptibility as determined by in vitro testing.

The decrease in the number of sensitive strains isolated from the urinary tract does not seem to be related entirely to previous antibiotic treatment in a given individual.

Because of the importance of obstruction to urine flow as a factor in the pathogenesis of genitourinary infections, the urinary tract is an ideal situation for the development of a bacterial flora resistant to antibiotic agents.

We wish to express our appreciation to Dr. J. Hartwell Harrison and his staff, who made the recent cultures available.

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CHROMOGENIC ACID-FAST BACILLI

A Bacteriologic and Clinical Study

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CHROMOGENIC acid-fast bacilli are organisms which develop a distinct pigment upon culture. They are of questionable pathogenicity for men and experimental animals.[1-5] In the past year, cultures of 850 specimens were made for acid-fast bacilli in this laboratory; 16 of these grew chromogenic organisms.

The isolation of such organisms always leads to confusion, especially when found in a patient suspected of having tuberculosis or when isolated from a proved case of tuberculosis in a patient who has received antituberculosis therapy. Studies are therefore indicated to establish whether or not these organisms are pathogenic.

To discuss tuberculosis an understanding of virulence is essential.[6, 7] It includes a knowledge of the relationship of the dosage of the organism and the variable factors of host resistence. The type of disease produced must also be considered.

In experimental tuberculosis virulence is manifest by the ability of the organism to produce constantly a progressive or fatal disease in animals. The chromogens have been noted to produce disease in the guinea pig and have been recovered from this animal after three weeks.[5] They can even kill the guinea pig.[3, 4] But, in contrast to the true tubercle bacilli, the chromogens do not invariably produce progressive and fatal disease in experimental animals. Pinner[3] noted difficulty in detecting chromogens in tissue as the time between injection and necropsy increased.

Pigmentation has been utilized as a morphologic means of differentiating mycobacteria. Griffith[8] was successful in utilizing media to alter pigmentation. He noted that human strains developed rich yellow-orange pigment on golden yellow bovine serum while bovine strains did not become chromogenic.

MATERIALS AND METHODS

Bacteriologic studies were performed on: thioglycollate broth (Difco); heart brain infusion (Difco); prepared slants of Petragnani medium (Difco) and Loeffler blood serum (Difco); and a modification of the tuberculin formula of the Bureau of Animal Industry.[9] All media were incubated at 37° C. In addition Petragnani medium was used to test growth at room temperature (average 24° C.).

A total of 31 strains of bacteria were tested, of which 3 were established saprophytes, *M. butyricum, M. phlei,* and *M. smegmatis.** Two were known virulent tubercle bacilli, strain H 37 Rv* and F (a strain from our laboratory). The avirulent strain H 37 Ra* was

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*Obtained through the courtesy of Dr. R. A. Patnode, Communicable Disease Center, Chamblee, Ga.

TABLE I. CASE SUMMARY

NO.	NAME	SEX	AGE	FINAL DIAGNOSIS	SOURCE OF CULTURE	P.P.D. #1	ANTI-TBC THERAPY	OUTCOME
1	D. K.	F	29	Pul. TBC	Gastric washing		STM INH	Under treatment for TBC
2	D. B.	F	25	Pul. and bone TBC?	Pus from ankle	+	None	Well
3	W. Mc.	M	78	Carcinoma of bladder	Urine		None	Living with no recurrence of carcinoma
4	J. K.	M	66	Congestive heart failure	Pleural fluid	–	None	Well
5	M. M.	F	28	Atypical pneumonia	Gastric washing		None	Well
6	E. L.	F	47	Adenocarcinoma of lung	Sputum	+	None	Dead. No findings of TBC at autopsy
7	A. H.	M	?	Pul. TBC	Gastric washing		STM INH	Dead (pul. TBC)
8	R. Q.	F	?	Atypical pneumonia	Sputum	+	None	Well
9	G. A.	F	?	Pul. TBC	Gastric washing		?	Under treatment for TBC
10	V. M.	M	55	Pul. TBC	Sputum		STM* collapse	"Arrested" TBC
11	H. C.	F	?	Pul. TBC	Gastric washing		?	Under treatment for TBC
12	L. W.	F	13	Abscess	Pus from abscess	–	STM (1 week)	Well
13	K. H.	F	57	Cystitis, PID, "arrested" TBC	Urine		None	Persisting symptoms of PID
14	B. M.	F	35	Atypical pneumonia	Sputum		None	Well
15	M. B.	F	40	PID	Urine	–	STM INH	Well
16	M. S. B.	F	29	Pul. TBC	Gastric washing		STM INH*	Under treatment for TBC

Key: STM = streptomycin; INH = isoniazid; PID = pelvic inflammatory disease.
*Therapy begun prior to isolation of chromogen.

also studied. The remaining 25 constitute the "chromogen" group. Nine were established chromogens.* All 25 were studied in vitro and 10 of the 25 were studied in vivo. Six of our chromogens were obtained too late to be included in the in vivo studies (see Table I).

Because many investigators have had success infecting the mouse with tubercle bacilli,[10-13] these animals were selected for virulence studies. Specifically, the dilute brown, nonagouti, line 1 dba was chosen since Pierce and associates[10] found it highly susceptible to tubercle bacilli injected intravenously. Bloch[14] found that young cultures of acid-fast bacilli produced microscopic lesions. He achieved excellent results with 3-week-old cultures.

Three-week-old cultures grown on the modified medium of the Bureau of Animal Industry were centrifuged in a Hopkins tube and the sediment was washed with saline solution, recentrifuged, and adjusted in saline solution to the 1:50 dilution. Into 4 or 5 mice between 4 to 6 weeks of age 0.5 ml. was injected intravenously.

The neutral-red cytochemical test of Middlebrook and Dubos[15] was used in an effort to determine the virulence of the acid-fast bacilli in vitro.

RESULTS

All strains grew on Petragnani medium (see Table II). The saprophytes and Strain 12 grew the most rapidly and luxuriantly, appearing overnight.

*Obtained through the courtesy of Dr. M. S. Tarshis, Veterans Administration, Memphis, Tenn.

TABLE II. BIOLOGIC AND CULTURAL PROPERTIES OF CHROMOGENS ISOLATED IN THIS LABORATORY AND OF OTHER ACID-FAST ORGANISMS USED FOR COMPARISON

STRAIN NUMBER	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	A	B	P	S	V	F
Petragnani																						
37° C	6	5	4	5	4	4	5	3	4	4	9	1	9	5	5	17	5	1	1	1	4	7
24° C	28	8	5	6	15	5	6	8	5	5	10	3	11	10	21	19	–	5	5	5	–	–
Loeffler blood serum	15	6	9	8	17	19	19	–	16	13	19	2	–	–	5	–	4	1	1	1	8	10
Thioglycollate	18	10	10	–	16	–	10	–	–	–	11	2	–	–	12	–	–	2	2	2	–	–
Pellicle formation*	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+	+	+	–	–
Neutral red test*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+

The nine chromogens kindly submitted by Dr. Tarshis had a pattern of growth essentially similar to the one described in his paper with Frisch.[4]

Key: Numbers represent days after which growth was first observed.
– stand for negative or no growth.
+ stands for positive.

*Pellicle formation was tested on the three following media: thioglycollate broth, heart brain infusion, and Smith's modification of the synthetic broth used for tuberculin production.

Strain of bacterium:

A—H 37 Ra
B—*M. butyricum*
P—*M. phlei*
S—*M. smegmatis*
V—H 37 Rv
F—*M. tuberculosis* (vir.) isolated in this laboratory

Period of observation: 30 days. Subcultures studied.

The saprophytes were discrete, heaped, rough, lightly pigmented, yellow colonies. The three tubercle bacilli took one week to develop after subculture. The chromogens also grew slowly, but showed a wider range of development—the earliest appearing at three and the latest at twelve days. They were, with the exception of Strain 1, moist, confluent, convex, flat, and yellow to orange in color. The initial pigment became more intense with age. The growth of Strain 1 was dry and the colonies were discrete and rough, resembling the colonies of the tubercle bacilli except for the deep yellow-orange pigment.

On Loeffler's blood serum the saprophytes and Strain 12 grew easily. Several of the chromogens did not grow. The other chromogens and the tubercle bacilli grew slowly. All were morphologically identical with, but smaller than, the colonies on Petragnani medium. With the exception of Strain 1, all the chromogens were pigmented on Loeffler medium; Strain 1 resembled the whitish color of the medium. The colonies of the known tubercle bacilli were also whitish and indistinguishable from Strain 1.

In thioglycollate broth the saprophytes and Strain 12 grew easily and formed thick coarse pellicles. The tubercle bacilli did not develop, nor did many of the chromogens. The chromogens that grew developed in a fine latticework pattern on the bottom of the test tube; Strain 1, alone, formed a thin, incomplete, satiny pellicle.

In brain heart infusion growth of the chromogens was very poor. A few grew slowly on the bottom of the test tube. The saprophytes and Strains 1 and 12 had a growth pattern similar to that in thioglycollate. The tubercle bacilli did not grow.

At room temperature the tubercle bacilli were the only group not to grow. With the exception of Strains 1, 15, and 16, all the chromogens grew between 5 and 15 days. These exceptions required between 3 and 4 weeks for development. For all, the morphology typical of Petragnani medium was seen, although the growth was not as luxuriant as in the incubator.

The neutral-red test was positive only for the virulent tubercle bacilli. Several other strains of pathogens were tested and all were positive. *None* of the chromogens or H 37 Ra took up any dye.

The mice were not killed by any of the bacteria injected into them. Several died within the first days of being injected. These showed no gross pathology and were not further examined. The remainder were sacrificed between 5 and 6 weeks. Small grayish nodules of 1 to 2 mm. size were present in the lungs, liver, and spleen of the animals injected with suspensions of virulent tubercle bacilli and Strain 1. Sections examined with hematoxylin and eosin showed solitary and confluent granulomas. Examination of acid-fast preparations revealed acid-fast rods in the granulomas. We found, as did Pinner,[3] that experimental infection with the chromogens resulted in more and larger acid-fast bacilli in tissue than did similar infection with virulent tubercle bacilli. For all the remaining animals there was no macroscopic or microscopic evidence of disease.

Seven pairs of guinea pigs gave uniformly negative results (Table III) except for small nonspecific abscesses at the point of injection in two animals. No evidence of acid-fast infection was found on microscopic examination.

TABLE III. PATHOGENICITY STUDY OF TEN CHROMOGENS PATIENT LISTING

STRAIN	NAME	SOURCE	MOUSE	GUINEA PIG
1	D. S.	Gastric	+	–
2	D. B.	Ankle	–	*
3	W. M.	Urine	–	–
4	J. K.	Pleural fluid	–	*
5	M. M.	Gastric	–	–
6	E. L.	Gastric	–	–
7	A. H.	Gastric	–	Not done
8	R. Q.	Sputum	–	–
9	G. A.	Gastric	–	Not done
10	V. M.	Sputum	–	Not done

*Focal myositis at point of injection but no tuberculous tissue reaction on gross or microscopic examination.

CLINICAL DATA

Our chromogenic organisms were isolated from sixteen patients. The clinical nature of these allows for division into three categories: (1) cases of proved tuberculosis (Strains 1, 7, 9, 10, 11, 16), (2) cases suspected of having tuberculosis without definite proof (Strains 2, 13, 14, 15), and (3) cases where tuberculosis was neither suspected nor ultimately found (Strains 3, 4, 5, 6, 8, 12).

Group 1.—Of the six cases one patient apparently died of tuberculosis; one case is arrested and has been for the past several years. The remaining patients had active tuberculosis at the time of their positive chromogen culture and are at present under treatment for tuberculosis. In two of the patients antibiotic therapy was begun prior to the isolation of chromogens. In one (Case 7) information is not available.

CASE 1.—In May, 1953, this 30-year-old white woman complained of pain in the left breast but no connection with intrathoracic pathology could be established. A chest x-ray, however, revealed a small cavity in the left upper lobe. Only a slight wheezing could be heard over this area. The patient ran subfebrile temperature but otherwise remained asymptomatic. Blood counts and sedimentation rate were always within normal limits. Strain 1 was recovered in June, 1953, from 3 consecutive gastric washings taken during her stay in this hospital. A reportedly "typical" tubercle bacillus was recovered again from gastric washings a few days later in the tuberculosis sanitarium where the patient transferred. This strain was not tested for pathogenicity. The organism was never recovered again although repeated attempts were made. The patient was hospitalized for several months. The cavity became smaller and closed approximately 12 months after the initial culture was obtained. At present the patient is still asymptomatic and apparently well.

Group 2.—The four patients were diagnostic problems as illustrated by the following case history.

CASE 2.—A 25-year-old white woman hurt her ankle while bowling. A fistula developed from which the organisms were isolated. Shortly afterward unilateral pleural effusion developed from which no organisms could be recovered. On streptomycin therapy the patient had a rapid, apparently complete recovery with no permanent joint impairment. Her chromogenic strain was not pathogenic for animals. Bacteriologically it was similar to organisms from the nontuberculous patients. This woman, however, reacted to specific therapy and has a positive P.P.D. 1.

Group 3.—The six patients of this group did not have tuberculosis as proved by autopsy, negative tuberculin test, or follow-up. Two examples are given.

CASE 12.—A 13-year-old girl developed an abscess at the injection site of poison ivy extract. A chromogenic organism bacteriologically resembling a saprophyte was isolated.

On the basis of the presence of acid-fast bacilli on smear (demonstrated elsewhere), streptomycin was given for 1 week. Without further specific therapy the abscess subsequently healed slowly. P.P.D. 1 was negative on two occasions. A biopsy from the abscess showed nonspecific subacute cellulitis, but no acid-fast bacilli.

CASE 4.—This 66-year-old minister was seen with pleural effusion from which the organism was isolated. Before the results of the chromogen culture were known, digitalis therapy resulted in complete clearing of the effusion. P.P.D. 1 was negative several months later. Follow-up for more than two years failed to show evidence of any tuberculosis.

In Group 1 chromogens were isolated from gastric washings in five patients and from sputum in one.

In Group 2 the cultures were obtained from urine in two patients, and once each from sputum and pus from a draining sinus.

In Group 3 positive cultures grew from gastric washings of two patients and once each from urine, pus, sputum, and pleural fluid.

DISCUSSION

The data at hand suggest that the acid-fast bacilli studied fall in one of four groups:

1. *"True Tubercle Bacilli."*—These are pathogenic even in very small number and are dry and slow-growing on culture. They do not develop at room temperature and do not produce pellicles in liquid media. They give a positive neutral-red test.

2. *"Saprophytes."*—These are pathogenic only in enormous doses and generally produce self-limited lesions. They grow fast even at room temperature and are generally dry in appearance, *produce* a pellicle, and are negative in the neutral-red test.

3. *"Chromogens."*—They are pathogenic only in enormous doses, are moist, fairly fast-growing, orange-yellow pigmented colonies even at room temperature. They *do not* produce a pellicle and have a negative neutral-red test.

4. *"Atypical Pathogenic Strains."*—Our Strain 1 was pathogenic for mice. It grew in dry, yellow-orange colonies, produced a very thin, incomplete pellicle, and grew slowly at room temperature. It repeatedly lost its pigmentation on Loeffler blood serum (subcultures on Petragnani medium were pigmented). The neutral-red test was negative.

Two possibilities are suggested by the information available. One is that the chromogens are completely coincidental, nonpathogenic organisms found in cases of tuberculosis. The second is that the chromogens are mutant forms of virulent tubercle bacilli, as has been suggested by Schiff and Tarshis.[16] The antibiotics used in some of the cases of active tuberculosis could conceivably be the agents responsible for the mutation. Two of our strains would not support this assumption. Case 1 had a strain that acted like a virulent tubercle bacillus, in vivo, but was chromogenic when isolated. Case 10 had several positive cultures of typical virulent tubercle bacilli despite antibiotic therapy (streptomycin) up to mid-1950. He has received no antibiotic therapy since, and until the time of his positive chromogen culture in 1953, numerous cultures failed to show acid-fast bacilli. No cultures have been positive since.

In favor of the organisms being purely coincidental findings is the following fact: Chromogens were present in six persons without clinical evidence of tuberculosis and in six with tuberculosis.

It would, therefore, appear that these organisms represent bacteria which in every instance must be tested for pathogenicity in animals. Further clinical studies are needed to establish the role of all such chromogenic acid-fast bacilli in human disease.

SUMMARY AND CONCLUSIONS

A clinical and bacteriologic study of sixteen strains of chromogenic acid-fast organisms was made. The strains were recovered in the Clinical Laboratories of the Jewish Hospital during the year 1953 from 850 routine acid-fast cultures.

In vivo studies were made using susceptible mice and injecting the organisms intravenously. Guinea pigs were injected subcutaneously and observed for six weeks. Cytochemical testing was done using the neutral-red test of Middlebrook and Dubos.

In the period of observation there were no indications of progressive disease developing in mice except for Strain 1. The neutral-red test was negative with chromogenic acid-fast bacilli.

Clinically, insufficient evidence was available to draw definite conclusions. These organisms are found in man with and without clinical tuberculosis. Demonstration of chromogenic acid-fast bacilli is no proof of tuberculosis. However, finding them should always prompt pathogenic studies in susceptible animals. Nonpathogenic strains are not acceptable as laboratory evidence for the presence of clinical tuberculosis.

Chromogenic acid-fast bacilli produce relatively fast-growing, moist, yellow-to-orange colored colonies. Large numbers of bacilli inconsistently produce disease in animals. They grow at room temperature and do not produce pellicles. The neutral-red test is negative.

Atypical pathogenic bacilli even if pigment producing can be differentiated from chromogenic (nonpathogenic) bacilli by animal inoculation.

Nine out of ten pigment-producing mycobacteria in this series proved to be nonpathogenic for mice. It is a serious mistake to report such cultures as "tubercle bacilli" without additional investigation.

A positive test in susceptible mice as contrasted with the negative results in guinea pigs illustrates the (at present) unsurmountable difficulty of classification of mycobacteria for clinical use (Strain 1).

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STUDIES ON ISOSENSITIZATION TO THE KELL FACTOR

A Report of One Case

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IN 1946 Coombs, Mourant, and Race[1] discovered the Kell blood group. In 1950 seventeen cases of isosensitization to this blood factor reported by several investigators were summarized by Race and Sanger.[2] Since then fifteen other examples of the Kell antibody were identified.[9-11, 14-20, 27, 29] Up to the present time ten cases of hemolytic disease of the newborn infant caused by maternal isosensitization to the Kell factor have been described,[1, 3-11] and eleven transfusion reactions chiefly due to sensitization to the same blood factor have also been observed.[11-21] Nine serum samples containing anti-Kell were reported[6, 22, 29] without revealing the cause of antibody formation, and two samples of volunteers who had successfully been immunized were made known.[6, 27] In several instances anti-Kell was found together with another antibody.[2, 11, 13, 16, 17, 19, 20, 29]

These reports indicate that isosensitization to the Kell factor is not very common due to the evidently low frequency of this blood factor. The effects, however, resulting from isosensitization to the Kell factor resemble those due to Rh isosensitization and no difference appears to exist between the clinical manifestations arising from isosensitization to either blood factor. On the other hand, isosensitization to the Kell factor is becoming better recognized as a cause of severe hemolytic transfusion reactions because of the constantly increasing number of patients transfused as well as the larger amount of blood more frequently administered to the individual patient. The purpose of this presentation is to report the results of further investigation on isosensitization to the Kell factor. The following case deals with another example of anti-Kell recently discovered in a female patient who had repeatedly been transfused. The management of this case is best illustrated by measures taken to eliminate the possibility of hemolytic transfusion reactions.

CASE REPORT

Mrs. I. C., 40 years of age, white, had been a healthy woman until 1941 when at a New York hospital a growth in the right lumbar region was diagnosed as a hemangio-endothelioma. An unsuccessful attempt at removal of this tumor was accompanied by blood transfusions. Several months later at another hospital a similar attempt had to be abandoned because of uncontrollable bleeding. During this operation she received numerous blood transfusions until the surgeon was successful in tying a number of lumbar arteries and veins which fed the mass. As a result the tumor shrank considerably and the patient appeared free of symptoms although x-ray films showed destruction of the second and third lumbar vertebrae.

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In 1948 at this hospital she was delivered of a male infant by cesarean section. She had to receive again two blood transfusions because of severe uterine hemorrhage resulting from a placenta previa. In 1951 she was readmitted to the aforementioned New York hospital for a work-up, preliminary to a possible attempt at excision of this mass. The thought of operation was, however, abandoned because it was felt that the mass was inoperable, and she was also advised to have no more children. In September, 1953, however, she gave birth to another infant at this hospital. Since then destruction of the adjoining vertebrae by this tumor progressed, and root nerve symptoms of both lower extremities developed. On May 13, 1954, the patient was, therefore, readmitted to this hospital for corrective surgery.

RESULTS

Serologic Analysis.—Examination of the patient's blood showed her to belong to Group B, Type Rh_1. In view of her past transfusion history, the possible presence of isoantibodies against some of the newer blood factors was taken into consideration. Tests could be immediately carried out with cells of specific antigenicity for the factors Kell (K), Duffy (Fy), and S on hand, and revealed that this patient's serum contained a potent Kell antibody demonstrable by the indirect antiglobulin technique. All tests yielded a strongly positive reaction. Further blood group studies disclosed the patient as group B,Rh^w_1rh,MS, P,kk,Fy(a+). It became clear that this patient being Kell negative presented an example of isosensitization to the Kell factor. One out of nine units of bank blood crossmatched with her serum by the antiglobulin technique appeared to be incompatible, this unit being Kell positive (Table I). From the eight units of blood found compatible she received four units (29109, 29137, 29112, 29178) during the operation, and another unit of blood on the eleventh and fourteenth postoperative days, respectively, without any unfavorable response.

TABLE I. BLOOD GROUPS OF THE PATIENT, HER HUSBAND, THE DONORS CROSS-MATCHED FOR THE PENDING OPERATION, AND THOSE DONORS BACK FROM 1948

	ABO	M-N	Rh	Fy^a	S	P	ANTI-KELL SERUM*	PATIENT'S SERUM
Patient	B	M	Rh^w_1rh	+	+	+	0	0
Her husband	B	M	rhrh	+	+	+	0	0
Donors cross-matched:								
29178	B	MN	Rh_1Rh_1	+			0	0
29118	B	MN	Rh_1Rh_1	+	-	-	Positive	Positive
29137	B	MN	Rh_1Rh_1	-			0	0
29112	B	MN	Rh_1rh	-			0	0
29090	B	M	Rh_2rh				0	0
29081	B	N	Rh_1rh				0	0
29085	B	MN	Rh_0rh				0	0
29098	B	M	Rh_1Rh_1				0	0
29109	B	MN	Rh_1rh				0	0
Donors back from 1948:								
11390	O	M	Rh_1Rh_2	+	+	+	Positive	Positive
11422	B	MN	Rh_1rh	+	+	+	0	0

The symbol "0" used in the last two columns indicates a negative reaction.
*Obtained from Certified Blood Donor Service, Jamaica, N. Y.

Pregnancy as the source of sensitization to this blood factor could be excluded when tests on her husband showed that he was Kell negative (gene type kk). Evidence could, however, be established that the patient had become sensitized to the Kell factor by blood transfusions when tests carried out on those two individuals who, fortunately, could be traced as the donors of 1948 revealed that one of them was Kell positive (gene type KK or Kk). Among thirty-seven selected blood specimens belonging to Group O and B, two were also found to be of type K (KK or Kk). The presence of other antibodies was finally ruled out in repeated tests of the patient's serum against panels of red cells of known antigenic structure.

The antibody exhibited optimal activity at 37° C. and could not be destroyed at 56° C. when a serum sample accidentally was left in the water bath for more than six hours. The undiluted serum occasionally weakly agglutinated type K cells in saline solution, but was found to clump strongly the same cells by the indirect antiglobulin technique. Table II presents the titration experiments in which the indirect antiglobulin technique, the plasma-albumin method, and trypsinated red cells were employed. While the indirect antiglobulin technique usually gave clear-cut results yielding a titer of 64 to 128 units, the same cells sensitized by this antibody agglutinated much weaker in the albumin media showing a titer of 8 to 16 units in strength. The titer obtained with trypsinated red cells was 2 to 4 units. This titer could be restored to its original strength when the indirect antiglobulin method was repeated in these tests,[24] thus demonstrating that the enzyme treatment of red cells did not decrease sensitivity of the Kell factor (Table II). The tests were carried out as follows: Packed cells of type K and type kk washed four times with saline solution were treated with an equal volume of a 1 per cent trypsin solution for 1 hour at 37° C., and then washed once and incubated with the patient's serum for 1 hour at 37° C.; after reading of the titer the antiglobulin test was performed.

TABLE II. TITRATION OF KELL ANTIBODY IN SERUM OF PATIENT

	REACTION OF SERUM DILUTIONS BY FOLLOWING TECHNIQUES			
BLOOD CELLS	INDIRECT ANTIGLOBULIN	PLASMA-ALBUMIN	TRYPSINATED CELL	TRYPSINATED CELL INDIRECT ANTIGLOBULIN
Known type K 29118:	1:64	1:16	1:2	1:64
Incompatible donor 11390:	1:128	1:8	1:4	1:128
Incompatible donor of 1948 29178:	1:128	1:16	1:4	1:128
Compatible donor	-	-	-	-
Known type kk	-	-	-	-

Studies on the incidence of the Kell factor were carried out. For this purpose the serum was neutralized. An unselected group of 505 individuals was examined. The bloods originated from 301 Caucasians and 204 Negroids, all residents of Newark and surrounding areas in Essex County. Among the

Negroids, dark-colored subjects were selected; the degree of racial admixture, however, could not be determined. All tests were performed within several hours after the blood specimens were obtained. The figures of frequency distribution for Caucasians and Negroids presented in Table III appear similar to those established by other investigators.[7, 12, 16, 28]

TABLE III. THE FREQUENCY DISTRIBUTION OF THE KELL FACTOR AMONG AN UNSELECTED GROUP OF 505 INDIVIDUALS CONSISTING OF 301 CAUCASIANS AND 204 NEGROIDS

CELL TYPE	REACTIONS WITH PATIENT'S SERUM	FREQUENCY (%) IN NEWARK AND ESSEX COUNTY	
		CAUCASIANS	NEGROIDS
KK and Kk	Positive	11.63	1.47
kk	Negative	88.37	98.53

DISCUSSION

In the majority of the cases so far reported the presence of anti-K has been associated with a hemolytic transfusion reaction or with clinical manifestations of hemolytic disease of the newborn infant. The case described presents an example of isosensitization to the Kell factor in which a possible transfusion reaction could be prevented by testing the patient's serum against cells of known antigenic structure for the possible presence of isoantibodies. Pregnancy as a cause of sensitization to this blood factor could be ruled out from the standpoint that the husband was found to be Kell negative. There was no possibility to test the presence or absence of the Kell factor in the patient's children. Extramarital relations as a possible etiological factor in this immune process were not likely to be involved. Her transfusion history did not reveal to what extent she had become sensitized to type K. It was impossible to test those donors who gave blood to the patient in 1941. Information obtained from her physician revealed that she received during both operations ten blood transfusions among which one transfusion may or may not have been of type K. Assuming that one transfusion was of type K, the first contact with the Kell antigen had become established at that time. According to Wiener,[11] that transfusion presumably "primed" the patient. In 1948 the patient received a transfusion of proved type K blood which was followed by a marked rise in temperature (102.6° F.) for the next two consecutive days. Although it was not definitely established that one of those transfusions in 1941 presented the first exposure to the Kell factor, this transfusion was regarded as the second one which stimulated antibody formation without precipitating the manifestations of a hemolytic transfusion reaction. Six years later, when the patient was readmitted to this hospital and the administration of blood during surgery was contemplated, a high Kell antibody titer could be discovered in her serum.

Titer studies exhibited the characteristic serologic pattern of the Kell antibody; remarkably good reactivity by the indirect antiglobulin technique and contrasting poor response to the agglutinogen when albumin media or trypsinated red cells were employed in the test. The trypsinated cell indirect

antiglobulin method did not yield that sensitivity found by Unger[24] in similar experiments with sera containing Rh_0 antibodies of extremely low titer.

Clinical observations do not indicate that the Kell factor is a weakly reacting blood factor. This point of view is supported by some investigators[14, 25] who suggested that the Kell factor is almost as antigenic as the Rh_0 factor, while others[26] compared its potency with the factor hr′. Race[27] subjected each of three Kell-negative individuals to two intravenous injections of Kell-positive blood at an interval of three months. Two weeks after the second injection one of the three appeared to have become sensitized to the Kell factor, thus demonstrating its well-functioning antigenicity in transfusions. Mollison[14] calculated from the remarkably lower incidence of the Kell factor that sensitization to this blood factor occurs 100 times less often than to the Rh_0 factor, assuming equal antigenicity for both. From this point of view, Wiener[11] explained the relative infrequency of hemolytic transfusion reactions due to Kell sensitization, emphasizing that three contacts with the antigen are required before such reactions are likely to result. On the other hand, Mollison[14] could show that "it is very unusual for a person having three spaced transfusions to receive Kell-positive blood on each occasion"; according to Wiener,[12] the probability of such transfusions is 1:1,000. The case reported could have easily been turned into such a rare instance had specific compatibility tests been omitted and Kell-positive blood probably would have been administered to this patient for a third time.

Ottensooser and associates[18] reported a fatal transfusion reaction due to Kell sensitization in a patient who received 500 ml. of type K blood without having been subjected to any compatibility test. This patient, who had become sensitized to type K by blood transfusions many years ago, would probably be alive had her past transfusion history been paid proper attention. The case presented here should, therefore, appear of special interest because of the preventive measures pointed out against hemolytic transfusion reactions.

Since the discovery of anti-k (Cellano) by Levine[23] in 1949, the genetic relationship of the Kell and Cellano factors appears, today, to be an established fact. Shapiro[16] designated the genes K and k as "co-dominant alleles" and pointed out their independent segregation from other blood group systems. Chown[30] has stressed the ethnologic significance of isoantibodies against the newer blood group factors including those designated as "private." He suggested always to inquire into the racial origin of the "stimulator" of an antibody defining a "family" blood factor, because its character may sometimes depend upon the population studied with such antibody. In an American Indian woman he found an antibody defining a blood factor as "private"; later, when her serum was tested exclusively against bloods of white people, this antibody was found to be anti-Kell.

SUMMARY AND CONCLUSIONS

A white, female patient is presented who had repeatedly been transfused in the past. Thirteen years ago she presumably received some Kell-positive

blood; six years ago, proved type K blood had been administered to her. The result was the formation of an antibody against the Kell blood factor. On her recent admission, preparatory to an operation, this antibody was discovered by the indirect antiglobulin technique using red cells of specific antigenic structure. A possible hemolytic transfusion reaction could thus be prevented.

The indirect antiglobulin technique is the method of choice for the detection of antibodies defining the Kell blood factor.

Established figures of frequency distribution for the Kell blood factor appear similar to those found by other investigators.

The suggestion is made to strive for better safeguards against unsuspected transfusion reactions. The proper concern of everyone who administers blood should be to adopt improved techniques and keep pace with the advances made in this field of medical science.

I wish to express my sincere thanks and appreciation to Dr. Alexander S. Wiener, Brooklyn, New York, for reviewing the serologic data of this case.

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THE USE OF HORSE KIDNEY IN THE DIFFERENTIAL TEST FOR INFECTIOUS MONONUCLEOSIS

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HETEROPHILIC antibodies can be defined[1] as antibodies that have the ability to react with antigens that are apparently unrelated to those that stimulated their production; heterophilic antigens are those capable of stimulating the production of such antibodies. The phenomenon of heterophilic antigen-antibody reactions was first described by Forssman[2] in 1911. He found that suspensions of tissues of the guinea pig, cat, and horse, when injected into rabbits, produced lysins of high titer against sheep erythrocytes. His work was amplified and expanded by many others, and the subject was reviewed by Taniguchi,[3] Davidsohn,[1] Buchbinder,[4] and others.

Forssman antigen is widely distributed. It is found in various tissues of the guinea pig, cat, dog, mouse, and horse and in erythrocytes of sheep and of man of blood groups A and AB.[5, 6] It also occurs in chickens, fish, and other animals[4] and in various species of bacteria such as the pneumococcus and *Shigella dysenteriae*. It is not found in the organs of the rabbit, ox, rat, pig, sheep, or man. In some species which contain heterophilic antigen in their erythrocytes it is absent in their tissues; in others, it is found in both locations. Although qualitative variations may exist, the Forssman antigen practically always occurs in the same organs of a given species and in each individual of a given species.

The Forssman antigen is not a single specific homogeneous entity. Its heterogenetic relationships depend probably upon the existence of similar rather than identical structures, which at present cannot be chemically identified. Thus, it is best considered as a serologic concept covering a group of substances which, when injected into rabbits, produce hemolysins or agglutinins for sheep erythrocytes.

Davidsohn[7, 8] observed that there was an increase in antisheep hemolysins and more particularly in agglutinins in the serum of persons suffering from serum sickness following administration of horse serum. There was no increase in titer of hemagglutinins for the erythrocytes of the rabbit, and the sheep cell antibodies could be removed by absorption with extracts of guinea pig kidney. Paul and Bunnell[9] and Bunnell,[10] in the course of checking these observations, observed a marked increase in titer of sheep hemagglutinins in

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the serum of four patients with infectious mononucleosis. Because the Forssman antigen was known to produce in man agglutinins against sheep erythrocytes, it was assumed that the antibodies in infectious mononucleosis were also of the Forssman type, i.e., absorbable with guinea pig tissue.

The original Paul and Bunnell or 'heterophilic antibody test'' is entirely nonspecific, since it is now known that antisheep agglutinins may occur in relatively high titers in normal persons and in individuals after horse serum injections and after various known and unknown antigenic stimuli.[7, 8, 12, 13]

Stuart and associates,[14, 15] Bailey and Raffel,[16] and Davidsohn and Walker[17] in investigations conducted almost simultaneously, but independently, showed that these agglutinins were not significantly absorbed by guinea pig tissues and by a wide variety of tissues of other animals containing Forssman antigen, but that sheep erythrocytes did absorb them. They concluded from these findings that the antibodies found in infectious mononucleosis are heterophilic, but not Forssman, in type. Davidsohn and Walker[17] and Davidsohn[18] showed: (1) that sheep erythrocytes contain both the Forssman antigen and a further heterophilic antigen which reacts with the heterophilic antibodies found in infectious mononucleosis, and (2) that absorption with guinea pig kidneys and with boiled beef cells could be used for differentiation of infectious mononucleosis from serum sickness and from other conditions associated with an elevated titer of antisheep agglutinins.

Table A, data adapted from Leibowitz[19] and Davidsohn and co-workers,[20] summarizes the differentiating characteristics of these heterophilic antibodies.

TABLE A

TYPE OF SERUM	HETEROPHILE ANTIBODY ABSORBED BY		OCCURRENCE OF ANTIGEN IN				
	G. P. KIDNEY	BEEF CELLS	SHEEP R.B.C.	BEEF R.B.C.	RABBIT R.B.C.	G. P. TISSUE	HORSE SERUM
Normal (Forssman)	Complete	Complete to partial*	+	−	−	+	+
Serum sickness	Complete	Complete	+	+	+	+	+
Infectious mononucleosis	Incomplete†	Complete	+	+	−	−	−

*Incomplete removal in about one-third of patients.

†Residual titer after absorption not less than 12 per cent of the original titer (expressed in twofold dilutions, the drop ranges from 1 to 3 tubes).

DIFFERENTIAL TEST

The differentiation of the heterophilic antibody of infectious mononucleosis from agglutinins for sheep red cells in various other conditions with the help of the so-called differential test is now almost universally accepted as a specific test. It is based on the absorption of the serum with two antigens: 20 per cent suspensions of guinea pig kidney and beef red cells. Properly performed, it leads to clarification of the true serologic nature of almost all instances in which infectious mononucleosis is considered.[11, 19, 21, 22] The technique for this ''differential test'' has recently been simplified and shortened by the senior author.[20]

It has been shown previously that the kidneys of the horse also contain Forssman antigen and, like the guinea pig kidneys, do not absorb the heterophile agglutinins of infectious mononucleosis. Davidsohn and Walker[17] found

that horse kidney suspensions did not absorb the sheep cell agglutinins from the serum of patients with diseases other than infectious mononucleosis as effectively as did the guinea pig antigen. They used a 20 per cent suspension and did not try more dilute suspensions. Since the horse kidney would have obvious practical advantages, it was thought worth while to reinvestigate the possibility of using this organ as antigen in place of the routinely used guinea pig kidney in the differential test for infectious mononucleosis.

MATERIALS AND METHODS

1. Fresh frozen horse kidney was thawed slowly at icebox temperature, washed thoroughly in cold physiologic saline solution until free of blood, and macerated in a Waring blendor. The emulsion was then suspended in saline solution and boiled in a water bath for one hour. The loss of volume on boiling was made up with sterile distilled water, and 0.5 per cent phenol was added. This is the same method as is used in preparing the guinea pig antigen used for the differential test for infectious mononucleosis.[23] Suspensions were made in physiologic saline solution in concentrations of 30, 20, 10, 5, 2, 1, 0.5, and 0.25 per cent.

2. Five serums from patients with clinical, hematologic, and serologic evidence of infectious mononucleosis and three serums containing antisheep cell agglutinins not related to infectious mononucleosis were tested. A "presumptive" test and a "differential" test after absorption with 20 per cent guinea pig kidneys, 20 per cent beef cells, and the varying concentrations of the horse kidney antigens were performed on each serum. The 1951 modification of the technique of the senior author[20] was adhered to closely throughout. Absorptions of serums were performed in the following manner.

In an 85 by 13 mm. test tube, 1 ml. of the thoroughly shaken antigen was placed; 0.2 ml. of inactivated serum was added and the mixture was shaken well and allowed to stand for three minutes. The tube was centrifuged at 1,500 r.p.m. for 10 minutes, and the supernatant carefully removed with a capillary pipette.

In ten 75 by 10 mm. tubes, 0.25 ml. of physiologic solution of sodium chloride was added to all tubes but the first; 0.25 ml. of the supernatant fluid was added to the first tube and an equal quantity to the second. This was mixed, 0.25 ml. transferred to the third tube, etc.; 0.1 ml. of a 2 per cent sheep erythrocyte suspension was added to all tubes, and the tubes were incubated at room temperature for two hours. Titers were considered to be the reciprocal of the highest dilution of serum which agglutinated the sheep cells. If no clumping was visible macroscopically, the tube was placed horizontally on the stage of the microscope and read with a 35 mm. objective and a 5× ocular. Results are listed in Table I.

TABLE I. EFFECT OF ABSORPTIONS WITH VARIOUS CONCENTRATIONS OF HORSE KIDNEY ON ANTISHEEP AGGLUTININ TITERS

SERUM NO.*	TITER BEFORE ABSORPTION (PRESUMPTIVE)	TITER OF SERUM AFTER ABSORPTION WITH									
		BEEF CELLS	20% G. P. KIDNEYS	SUSPENSIONS OF BOILED HORSE KIDNEY							
				30%	20%	10%	5%	2%	1%	0.5%	0.25%
1	448	0	112	0	28	56	56	112	112	112	112
2	224	0	112	7	14	28	56	112	112	112	112
3	448	0	448	X	X	X	448	448	448	448	X
4	896	7	896	X	X	224	448	896	896	896	X
5	896	7	896	X	X	X	448	448	896	896	X
6	56	0	0	0	0	0	0	0	0	0	7
7	56	0	0	X	X	0	0	0	0	0	28
8	56	14	0	X	0	0	0	0	0	0	7

X = Not done.
0 = No titer.
*Serums 1 through 5 were from patients with infectious mononucleosis; 6 through 8 were from patients negative for this disease.

3. From the preliminary series listed in Table I it became apparent that suspension of horse kidneys ranging from 0.5 per cent to 5.0 per cent gave, after absorption of the serums, results identical within one dilution to the 20 per cent guinea pig antigen. The 2 per cent suspension was then selected for testing a larger series of serums, although it appeared that the exact concentration was not critical. A series of 63 serums was studied over a period of 19 months; of these, 34 were from patients with clinical, hematological, and serologic evidence of infectious mononucleosis; 29 were negative for this disease. Results of this study are listed in Table II.

TABLE II. TITERS OF ANTISHEEP AGGLUTININS AFTER ABSORPTION WITH 20 PER CENT GUINEA PIG AND 2 PER CENT HORSE KIDNEY SUSPENSIONS

SERUM NO.*	TITER BEFORE ABSORPTION (PRESUMPTIVE)	TITER AFTER ABSORPTION WITH		
		BEEF CELLS	20% G. P. KIDNEY	2% HORSE KIDNEY
1	3,584	7	896	896
2	224	0	56	56
3	896	7	448	448
4	1,792	0	896	896
5	1,792	0	896	1,792
6	448	0	448	448
7	448	0	112	56
8	898	0	224	448
9	112	0	28	56
10	896	0	112	112
11	448	0	224	224
12	448	0	112	112
13	224	0	112	112
14	448	0	448	448
15	896	7	896	896
16	896	7	896	448
17	3,584	0	896	896
18	224	0	56	56
19	448	0	112	112
20	896	14	448	448
21	448	0	224	224
22	112	0	14	14
23	7,184	14	896	1,792
24	1,792	0	448	448
25	448	0	112	112
26	448	0	224	224
27	1,792	0	896	1,792
28	1,792	0	896	896
29	56	7	14	14
30	3,584	0	896	896
31	1,792	0	448	448
32	28,672	7	7,168	7,168
33	28	0	14	14
34	1,792	0	448	448
35-54	0	0	0	0
55-56	56	14	0	0
57-59	7	0	0	0
60	224	0	0	0
61	28	14	0	0
62-63	56	0	0	0

*Serums 1 through 34 were from patients with infectious mononucleosis; 35 through 63 were from patients negative for this disease.

4. Dehydration does not seem to alter the sensitivity or specificity of the guinea pig antigen, and dehydrated antigen is widely used. In order to determine whether dehydration would alter the specificity of the horse kidney as compared with the boiled suspensions, a preliminary series of seven serums was tested as described previously. The antigen used for absorption was dehydrated and defatted horse kidney was prepared by the "VioBin"*

*Furnished through the courtesy of the VioBin Laboratories, Monticello, Ill.

process. Suspensions were prepared in physiologic saline solution with 0.5 per cent phenol added, and ranged from 0.015 per cent to 1.0 per cent, and tests were run as described previously. Results are listed in Table III.

TABLE III. EFFECT OF ABSORPTIONS WITH VARYING CONCENTRATIONS OF DEHYDRATED HORSE KIDNEYS ON ANTISHEEP AGGLUTININ TITERS

SERUM NO.	TITER BEFORE ABSORPTION (PRESUMPTIVE)	TITER OF SERUM AFTER ABSORPTION WITH								
		BEEF CELLS	20% G.P. KIDNEYS	SUSPENSIONS OF DEHYDRATED HORSE KIDNEY						
				1%	0.5 %	0.25 %	0.125 %	0.06 %	0.03 %	0.015 %
9	224	0	56	56	56	56	56	56	56	56
10	224	0	56	56	56	56	56	56	56	56
11	448	0	448	448	224	448	448	X	X	X
12	896	7	896	896	896	896	X	X	X	X
13	896	7	896	448	896	896	X	X	X	X
14	56	0	0	0	0	0	0	7	X	X
15	112	0	0	0	0	0	7	14	56	X

X = Not done.
0 = No titer.
*Serums 9 through 13 were from patients with infectious mononucleosis; 14 and 15 were from patients negative for this disease.

5. From the preliminary series listed in Table III, it was apparent that suspensions of dehydrated horse kidney ranging from 0.25 to 1 per cent after absorption of the serums gave results identical with the 20 per cent guinea pig suspension. The 0.5 per cent suspension was then selected for testing a longer series of serums, although here again the exact concentration did not appear to be critical. A total of 26 serums were tested as previously described; of these, 12 were from patients with clinical, hematologic, and serologic evidence of infectious mononucleosis; 14 were negative for this disease. Results of this series are listed in Table IV.

TABLE IV. TITERS OF ANTISHEEP AGGLUTININS AFTER ABSORPTION WITH 20 PER CENT GUINEA PIG AND 0.5 PER CENT DEHYDRATED HORSE KIDNEYS

SERUM NO.*	TITER BEFORE ABSORPTION (PRESUMPTIVE)	TITER AFTER ABSORPTION WITH		
		BEEF CELLS	20% G.P. KIDNEYS	0.5% DEHYDRATED HORSE KIDNEYS
1	1,792	0	896	896
2	448	0	448	448
3	896	7	448	448
4	1,792	0	896	896
5	448	0	112	112
6	224	0	56	56
7	224	0	56	56
8	448	0	448	224
9	896	7	896	896
10	896	7	896	896
11	896	7	224	448
12	56	7	14	28
13-15	7	0	0	0
16-22	0	0	0	0
23	28	14	0	0
24	56	0	0	0
25	112	0	0	0
26	224	0	0	0

*Serums 1 through 12 were from patients with infectious mononucleosis; 13 through 26 were from patients negative for this disease.

RESULTS

1. A suspension of boiled horse kidneys in concentrations ranging from 0.5 to 5.0 per cent gave results almost identical in every case with those obtained

after absorption with 20 per cent guinea pig kidneys (Tables I and II). It is felt that a one-tube difference in titer is insignificant and is inherent in the technique used. It is notable that the more dilute suspensions of the horse kidney gave more specific results than did the more concentrated ones; this may be due to the dilution of some interfering factor in the horse kidney tissue.

2. A total of 63 serums of patients positive or negative for infectious mononucleosis, after absorption with 2 per cent boiled horse kidneys, gave results substantially identical with those obtained after absorption with 20 per cent guinea pig kidneys. This suspension has retained its specificity after at least 22 months storage in the refrigerator; its stability consequently appears to be at least equivalent to that of the guinea pig antigen.

3. Dehydrated and defatted horse kidney suspension prepared in concentrations ranging from 0.25 to 1.0 per cent gave results identical to the 20 per cent guinea pig suspension. A total of 26 serums from patients positive or negative for infectious mononucleosis, after absorption with 0.5 per cent dehydrated horse kidney, gave results almost identical with those obtained after absorption with 20 per cent boiled guinea pig kidney antigens (Table IV). It is doubtful that the defatting process is necessary, since neither the fresh horse kidney nor the dehydrated guinea pig antigen, which is commonly used, is defatted. However, no tests were made with horse kidney antigen which was dehydrated but not defatted.

It was felt that the dehydrated horse kidney antigen would have no more advantage over the suspension of horse kidney than the dehydrated guinea pig antigen has over the suspension of guinea pig kidney. Actually, since the stability or the cost of the antigen would be no problem, it is our feeling that the boiled suspension would be preferable from the point of view of reproductibility, ease of preparation, less likelihood of denaturation of the protein, etc.

DISCUSSION

The value of the differential test for infectious mononucleosis has been proved over a period of many years. Nevertheless, it appears from a perusal of the literature and by personal observation that in some laboratories only the nonspecific presumptive test is done. From the experience reported here, it appears that a dilute suspension of horse kidneys could be substituted for the 20 per cent suspension of guinea pig kidneys. This antigen offers certain major advantages: guinea pig kidneys are small, weighing about 3 to 5 grams on the average, and the animals are relatively expensive. Horse kidneys are readily available and one kidney from this animal could supply enough antigen for many thousands of tests. Another advantage would be that the horse kidney suspension is dilute, so that when serum is added to the antigen for absorption and then centrifuged there is no difficulty in pipetting the requisite amount of the absorbed serum without including some solid antigen particles. Thus, there would be less chance of nonspecific physical absorption simulating true absorption, and also the agglutination end points would be easier to read.

Finally, because one horse kidney could be used to make a great amount of antigen, these antigens would be uniform in composition rather than being prepared from large numbers of animals.

SUMMARY

Horse kidney can be substituted for the guinea pig kidney antigen used in the differential test for infectious mononucleosis. The optimal concentration appears to be 2 per cent of the boiled suspension and 0.5 per cent of the dehydrated one. The advantages of this substitution are discussed.

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THE EFFECT OF SUBFEBRILE DOSES OF BACTERIAL PYROGENS ON SPLANCHNIC METABOLISM AND CARDIAC OUTPUT

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INTRODUCTION

THE study of splanchnic metabolism by use of the Bromsulphalein extraction technique of Bradley and co-workers[1] has usually given reproducible results in normal individuals as well as in individuals in various disease states. Occasionally, however, unexplained erratic deviations from the expected values are encountered, particularly following intravenous administration of various materials. Since fever-producing doses of bacterial pyrogens are known to increase hepatic blood flow and decrease Bromsulphalein removal by the liver,[2] the present study was designed to determine if smaller quantities of pyrogens are capable of altering splanchnic metabolism without the production of fever or other frank clinical evidence of a pyrogenic reaction.

METHODS

The pyrogenic material used throughout the study was Pyromen,† a purified pyrogenic polysaccharide obtained from *Bacillus pyocyaneus*. Initially it was found that 5 γ of this substance could be given intravenously without the production of a febrile response; therefore, this dose was used in all instances.

Twenty-one healthy hospital patients who were free of clinical evidence of hepatic and cardiac disease were selected for study in the fasting state. In three of these splanchnic metabolism was studied preceding and one and one-half hours following Pyromen. A single set of observations was made on eleven others, two to three and one-half hours after the drug was given. In the remaining seven patients, cardiac output was measured before and two hours after Pyromen. Oral temperature was measured at least twice during a thirty-minute period prior to Pyromen administration and once every ten to fifteen minutes thereafter.

Hepatic blood flow was estimated by the Bromsulphalein method of Bradley and associates.[1] With two exceptions, all flows were calculated from the average of four determinations of the arterial-hepatic venous Bromsulphalein difference at five- to ten-minute intervals. Bromsulphalein clearance in milliliters per minute per square meter of body surface area was derived from the ratio of the Bromsulphalein removal rate to the arterial Bromsulphalein concentration; the values were compared with those previously obtained in this laboratory on normal subjects[3] and expressed as per cent of normal. Splanchnic oxygen consumption was derived from the product of the hepatic blood flow and the average of two determinations of the arterial-hepatic venous oxygen difference. Paired samples for determination of glucose concentration were obtained with the Bromsulphalein samples and the average of these was used for calculation of the net splanchnic glucose production.[4] A single sample of right auricular blood was secured at the end of

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the study for estimation of the mixed arteriovenous oxygen difference. Blood oxygen content was measured spectrophotometrically by the method of Hickam and Frayser.[5] Glucose concentration was determined by the method of Nelson.[6]

Cardiac output was measured by the dye-dilution technique.[7] Five milligrams of Evans blue dye was injected rapidly into an antecubital vein and samples of arterial blood were collected during the ensuing forty seconds from the opposite brachial or femoral artery at two-second intervals. Serum dye concentration was determined on undiluted samples with a Beckman-D.U. spectrophotometer.[8] All samples were read at 620 and 540 mμ in order that correction for hemolysis could be made.[9]

In all patients the procedures were well tolerated and anxiety was minimal. There was no significant variation in pulse rate following Pyromen administration, but four patients complained of transient chilliness and headache one to one and one-half hours after the drug was given.

RESULTS

In the three patients studied before and one and one-half hours after pyrogen administration, there was a decrease in Bromsulphalein clearance but no other consistent alteration. Observations on eleven patients made two or more hours after pyrogen administration showed striking changes from the

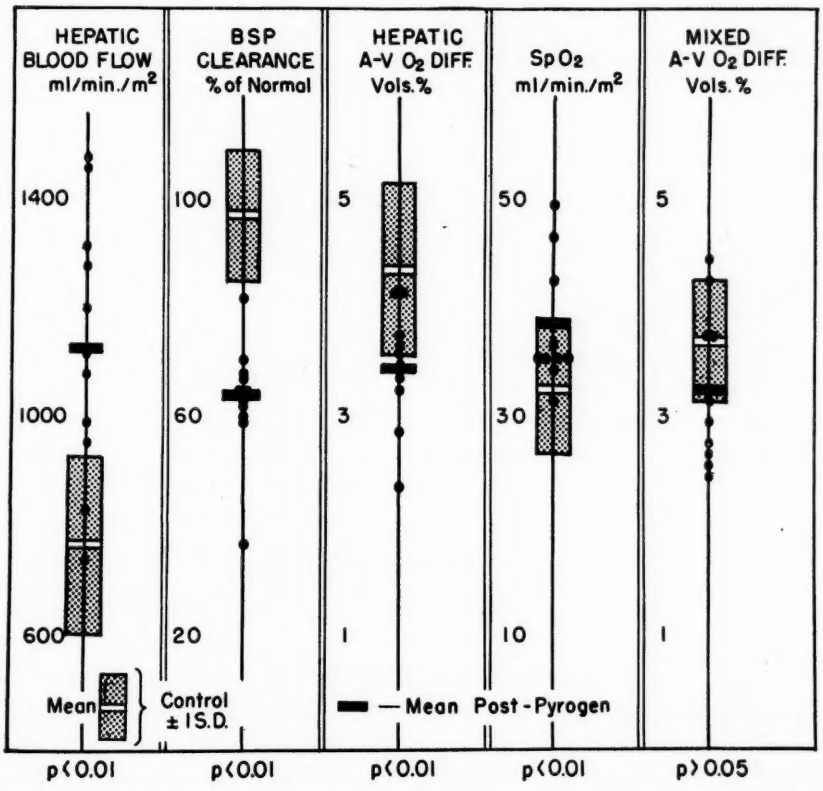

Fig. 1.—Splanchnic circulation and metabolism following subfebrile doses of bacterial pyrogens.

values obtained in a control series of sixty-three patients (Table I). This method of comparison was chosen in preference to performing control studies prior to pyrogen administration because of the technical impracticality of the more lengthy procedure.

TABLE I. SPLANCHNIC CIRCULATION AND METABOLISM 2 TO 3.5 HOURS AFTER PYROGEN

SUBJECT	AGE	SEX	TIME (HOURS) AFTER PYROGEN	BSP REMOVAL RATE (MG./MIN./M.²)	BSP ARTERIAL CONCENTRATION (MG. %)	BSP CLEARANCE (% OF NORMAL)	HEPATIC BLOOD FLOW (ML./MIN./M.²)	MIXED A-V OXYGEN DIFFERENCE (VOL. %)	HEPATIC A-V OXYGEN DIFFERENCE (VOL. %)	SPLANCHNIC GLUCOSE PRODUCTION (MG./MIN./M.²)	SPLANCHNIC OXYGEN CONSUMPTION (ML./MIN./M.²)	TEMPERATURE	
												CONTROL	POSTPYROGEN
M. S.	34	F	2.5	1.95	0.58	83	1,000	2.6	3.7	50	37	37.0	37.6
Z. H.	26	F	2.5	2.90	3.32	68	1,210	2.5	3.5	36	43	37.3	37.2
G. B.	34	M	3.5	1.96	1.39	61	840	3.8	4.2	37	36	37.5	37.4
L. D.	48	F	3.0	1.45	2.50	38	1,090	3.2	3.3	54	36	37.0	37.4
T. N.	44	M	2.0	2.15	1.80	63	1,130	- -	- -	85	--	37.0	37.6
T. B.	56	M	2.5	2.02	2.30	69	1,440	2.8	2.4	65	35	36.7	36.9
C. J.	18	M	2.5	1.75	0.74	66	1,240	3.8	2.9	68	36	36.9	36.6
R. B.	39	F	3.0	2.29	1.88	66	960	2.7	3.8	91	36	36.6	36.5
P. C.	33	F	3.25	2.06	1.35	64	750	4.3	4.2	97	32	37.3	37.3
C. M.	44	F	3.25	2.07	0.92	71	1,470	3.0	3.4	103	50	36.8	37.6
J. W.	52	M	2.25	1.75	0.92	60	1,320	4.5	3.6	79	47	37.0	37.6
Mean postpyrogen				2.03 ± 0.11*	1.61 ± 0.26	65 ± 3.2	1,132 ± 20	3.3 ± 0.23	3.5 ± 0.17	70 ± 7	39 ± 1.8		37.2 ± 0.12
Mean control						98 ± 1.5	772 ± 19	3.8 ± 0.12	4.4 ± 0.10	64 ± 3	33 ± 0.77	37.0 ± 0.08	
Mean difference						-33 ± 3.9	+360 ± 68	-0.43 ± 0.24	-0.88 ± 0.26	+8 ± 4	+6 ± 2.2		+0.24 ± 0.11
p value						<0.01	<0.01	>0.05	<0.01	>0.05	<0.01		>0.05

*Standard error.

As seen in Fig 1, there was a uniform decrease in Bromsulphalein clearance. The mean value following pyrogen was 65 per cent compared with a mean control value of 98 per cent. In all but one instance hepatic blood flow was increased by pyrogen, the average increase being 360 ml. per minute per square meter or +47 per cent. The arterial-hepatic venous oxygen difference fell moderately but significantly from 4.4 to 3.5 volumes per 100 ml. This fall was not proportionately as great as the increase in hepatic blood flow and, accordingly, splanchnic oxygen consumption was increased from 33 to 39 ml. per minute per square meter. The mixed arteriovenous oxygen difference, on the other hand, decreased slightly, but not significantly, from a mean of 3.8 to 3.3 volumes per 100 ml. There was no significant change in splanchnic glucose production. The average control temperature was 37.0° C. and during the determinations it was 37.2° C.; the range of variation was –0.3° to +0.8° C.

TABLE II. CARDIAC OUTPUT TWO HOURS AFTER PYROGEN

SUBJECT	AGE	SEX	CARDIAC INDEX (L./MIN./M²)		TEMPERATURE (°C.)	
			CONTROL	POSTPYROGEN	CONTROL	POSTPYROGEN
B. Si.	26	F	2.6	2.7	37.6	37.6
B. St.	28	M	6.8	6.1	37.2	37.6
L. M.	45	F	2.6	2.1	37.3	37.1
K. W.	15	F	4.9	4.8	37.5	37.4
E. G.	21	F	3.2	2.9	37.2	37.4
A. F.	41	F	3.7	4.0	36.6	37.7
J. F.	14	M	3.8	4.1	37.3	37.5
Mean			3.94± 0.57*	3.81± 0.52	37.2± 0.04	37.5± 0.08
Mean difference				-0.14± 1.51		+0.23± 0.16
p value				>0.3		>0.2

*Standard error.

The mean cardiac index of 3.81 L. per square meter two hours after pyrogen was actually slightly, but not significantly, lower than the control value of 3.94 L. per minute per square meter (Table II). This decrease was observed in four of the seven patients so studied and the maximum increase was only +9 per cent compared with the mean elevation of hepatic blood flow of +47 per cent. The range of temperature variation was similar to that noted in the previous group of subjects.

DISCUSSION

Striking increases in hepatic blood flow[2] and cardiac output[10, 11] have been shown to occur during the second hour following the intravenous administration of bacterial pyrogens even when the febrile response is blocked by antipyretics. Simultaneously, a marked decrease in Bromsulphalein excretion by the liver and an increase in splanchnic oxygen consumption have been observed.[2] No data are available, however, relative to the relation of the changes in cardiac output and hepatic blood flow to one another or to the effect of minute quantities of pyrogenic materials.

The results of the present study indicate that subfebrile doses of bacterial pyrogens produce alterations in splanchnic metabolism which, although similar to, are less pronounced than those following larger doses. In addition, since the increase in hepatic blood flow induced by subfebrile doses does not reflect a comparable increase in cardiac output, it must be compensated for by decreased blood flow elsewhere. These conclusions are borne out by the absence of significant change in either the mixed arteriovenous oxygen difference in the catheterized patients or the cardiac output as measured by the dye-dilution technique in a second group of subjects.

Contamination of parenterally injected materials with minute quantities of bacterial pyrogens is, therefore, offered as a possible explanation for erratic alterations in splanchnic metabolism. When such contamination has occurred, a marked decrease in Bromsulphalein excretion, a significant increase in hepatic blood flow, and a moderate elevation of splanchnic oxygen consumption should be evident.

SUMMARY

Subfebrile doses of bacterial pyrogens have been found to produce striking alterations in splanchnic circulation and metabolism. These consisted of a mean decrease in Bromsulphalein clearance of 33 per cent, a mean elevation of hepatic blood flow of 47 per cent, and a rise in mean splanchnic oxygen consumption of 18 per cent. Splanchnic glucose production was unaffected. The elevation of hepatic blood flow was not accompanied by proportionate increase in cardiac output. Contamination of parenterally injected materials with small amounts of bacterial pyrogens is, therefore, suggested as a possible cause for erratic alterations of splanchnic metabolism.

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PENTAQUINE AND QUININE IN THE TREATMENT OF KOREAN VIVAX MALARIA

A Controlled Study in 101 Patients

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A PRELIMINARY report from this hospital in January, 1952, indicated the considerable incidence of malaria caused by *Plasmodium vivax* in men returning from the conflict in Korea.[1] The clinical and laboratory findings were given in 25 cases. In addition, the immediate results of therapy were recorded. More recently a study of the relation of "liver function" tests to fever and therapy in 75 patients has been published.[2] There was no difference in the rate these tests returned to normal with either chloroquine or pentaquine* and quinine.

The present paper compares the immediate and remote results of two types of therapy, chloroquine and pentaquine-quinine. There are several papers in the literature on the use of pentaquine and quinine[3-5, 14-17] in vivax malaria. This is the first controlled study in naturally acquired, temperate-zone vivax malaria. The primary objective of the study was to determine whether pentaquine and quinine would suppress acute vivax malaria as effectively as chloroquine, yet prevent the relapses expected with chloroquine.

MATERIAL AND METHODS

During the three years ending May 30, 1954, 151 patients were admitted to the Veterans Administration Hospital, Minneapolis, Minnesota, because of Korean vivax malaria. Treatment was begun after the diagnosis was established by blood smears. The patients were assigned alternately to two therapeutic regimens. The present report includes 101 consecutive patients treated between May, 1951, and October, 1952. All the patients were young white males who were at bed rest in the hospital. Thick and thin blood smears usually were examined for *P. vivax* daily for four days during therapy and daily for three days after treatment was discontinued. The original antimalarial drug was chloroquine in 52 patients (Table I). Pentaquine and quinine were given to 49 patients as original treatment and to 4 other patients who had a relapse after chloroquine.† Chloroquine diphosphate was given by mouth, 1.0 Gm. initially and 0.5 Gm. daily for three days, or pentaquine, 10 mg., and quinine, 0.67 Gm., given orally three times daily for 14 days.

Nine patients given chloroquine returned to this hospital for recurrent malaria proved by blood smears. One additional patient treated with chloroquine returned several times for complications following splenectomy for infarction of the spleen. These were the only patients who were examined following their original treatment. On Aug. 4, 1953, a questionnaire was sent to all patients regarding further attacks of malaria. The questions

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*The pentaquine was generously supplied as pentaquine phosphate by Winthrop-Stearns, Inc., New York 18, N. Y.

†Three patients later were dropped from the pentaquine-quinine study because their treatment was incomplete.

included the number, dates, and duration of the recurrences; the presence of chills; the presence, degree, and duration of fever; the diagnosis and treatment by the attending physician; and any additional comments. Those patients who did not reply were sent a second questionnaire in November, 1953. A reply was received from 67 of the 101 patients, 35 of whom had been treated originally with chloroquine and 32 with pentaquine and quinine. The mean follow-up period was 19.4 months in subjects treated with chloroquine and 21.2 months in the others.

TABLE I. CLINICAL DATA IN 101 PATIENTS WITH KOREAN VIVAX MALARIA

	ORIGINAL TREATMENT	
	CHLOROQUINE	PENTAQUINE-QUININE
No. of patients	52	49
Av. time since in Korea (mo.)	4.8	4.5
Av. duration symptoms (days)	8.5	7.7
Palpable liver (per cent)	21.1	28.6
Palpable spleen (per cent)	71.1	67.3
Anemia, Hb. <12 Gm. per cent		
Before Rx (per cent)	9.6	17.0
During Rx (per cent)	7.7	14.3
Duration fever >98.6° F.		
>12 hr. (per cent)	32.0	52.2
Parasitemia during Rx (per cent)		
1 day	74.0	84.0
2 days	16.6	55.5
3 days	3.8	16.7
4 days	0.0	4.1
Complications (No.)		
Toxic psychosis	1	1
Drug sensitization	0	1
Splenic infarct	1	0
Hemolysis	2	3
Average stay in hospital (days)	9	17

RESULTS

Some of the important clinical data are given in Table I. The 101 patients were divided into two nearly equal groups and were comparable in time elapsed since leaving Korea, duration of symptoms, and incidence of palpable liver and spleen. The patients receiving pentaquine-quinine had a somewhat greater incidence of anemia before and during therapy. Chloroquine controlled fever and parasitemia more rapidly than pentaquine-quinine. The incidence of complications was low in both groups. A toxic psychosis, presumably caused by malaria, occurred in one patient in each group. One patient developed fever and a morbilliform rash caused by pentaquine on the eighth day.* Splenic infarction led to splenectomy in one patient on chloroquine. Evidence of hemolysis, including fall in hemoglobin, reticulocytosis, and increased fecal urobilinogen, was found in two patients receiving chloroquine and three on pentaquine-quinine. The average time in the hospital was nearly twice as long with pentaquine-quinine as with chloroquine.

The data on relapses are given in Table II. Sixty-seven of the 101 patients were followed for 13 to 30 months (mean = 20 months) after their original therapy. Of 35 patients treated with chloroquine, only 17.1 per cent had no further symptoms of malaria while 68.6 per cent of 35 patients given penta-

*There were no side effects which could be attributed to quinine or chloroquine.

quine-quinine had no relapse.* Nine patients returned to the hospital with a proved relapse after chloroquine but none of those on pentaquine-quinine returned.* Three patients given pentaquine-quinine for a proved relapse after chloroquine reported no further relapses; one failed to reply.

TABLE II. RELAPSES IN 101 PATIENTS WITH KOREAN VIVAX MALARIA

	TREATMENT		
	CHLOROQUINE	PENTAQUINE-QUININE	CHLOROQUINE, LATER PENTAQUINE-QUININE
No. of patients	52	49	4
No. followed after adequate therapy	35	32	3
Per cent with no relapse	17.1	65.6	100
		68.6	
Per cent with proved relapse	25.7	0	0
Per cent with possible relapse			
Physician diagnosis	34.3	3.1	0
Patient diagnosis	20.0	18.7	0
Recurrent fever	82.9	34.4	0
No. of relapses			
1	17	6	0
2	8	4	0
3	1	0	0
4	2	0	0
5 or more	0	0	0
Latent interval from primary attack to relapse (mo.)			
Minimum	0.5	2	-
Mean	3.3	3.7	-
Maximum	20	24	-

Although it is rather difficult to evaluate the possible relapses of malaria in patients followed only by questionnaire, recurrent chills and fever were noted with chloroquine more than twice as frequently as with pentaquine-quinine (Table II). Only one-fifth of the patients given pentaquine-quinine said they had relapses of malaria, while more than one-half those given chloroquine stated so. One-third of the patients on chloroquine had at least one relapse confirmed by a physician, about ten times the incidence in those treated with pentaquine-quinine.

The number of relapses in individual patients was no greater than four and usually was only one or two (Table II). The total number of relapses was more than three times greater in the patients given chloroquine than in those given pentaquine-quinine. The average interval from the end of treatment to relapse was about 3 months. The range was from 2 weeks to 24 months, but there were only 3 out of 58 possible relapses more than 12 months after therapy was completed. Nine patients who returned with a proved relapse after chloroquine had their recurrence from one to two and one-half months after treatment.

COMMENT

Korean malaria behaves as temperate-zone vivax malaria in that in many cases there is a long latent period.[7-9] Only 17 per cent of our patients had a definite previous attack of malaria in Korea or while traveling to this country.

*The difference is statistically highly significant, P = <0.0001.

The average interval since leaving Korea was about 4 months, but occasionally was as long as 10 to 11 months. The clinical features of the primary attacks were similar to those of tropical-zone vivax malaria (Table I). However, the number and severity of clinical relapses usually were considerably less (Table II).

The treatment of vivax malaria has been a subject of considerable recent interest. Many drugs are available which will kill the parasites in erythrocytes. Chloroquine diphosphate has been regarded by many as the best drug for the suppression and treatment of vivax malaria.[6, 10] Its low toxicity and prompt activity in small doses have led to widespread preference over both quinine and quinacrine (Atabrine). A high relapse rate has been its chief disadvantage.

Recently pyrimethamine (Daraprim) has been suggested as an ideal single-dose agent for the treatment of acute vivax malaria.[11] There have been reports indicating a small number of failures with a single dose of 25 to 50 mg. The immediate results seem to be nearly as favorable as with chloroquine but relapses are frequent.[12] Amodiaquin (Camoquine) is another new drug which promises to be very effective for the treatment of acute vivax malaria in a single dose of 600 mg.

The radical cure of vivax malaria requires the destruction of the tissue parasites as well as those in the erythrocytes. The drugs just cited will not do this. Pamaquine was one of the first drugs which seemed to have this property but it proved to be too toxic. Pentaquine was then offered as a safer substitute.[3] Also, pentaquine was found more active than pamaquine, primaquine, chloroquine, and quinine against experimental bird malaria.[3, 6]

Experience with experimental tropical-zone vivax malaria indicated that the proper dose was 60 mg. of pentaquine base daily.[3, 4] Pentaquine alone controlled fever and parasitemia satisfactorily, but relapse occurred with severe infections unless quinine was given concurrently. Only 3 of 17 patients with heavy infestations relapsed after pentaquine-quinine (Table III).[4] However, another similar study showed variable cure rates in different experiments.[13]

Monk[14] gave pentaquine alone in 25 cases of tropical vivax malaria. The average duration of fever was 1.2 days. The addition of quinine reduced the duration of pyrexia to an average of 0.73 days in 26 cases. A follow-up study 6 months later disclosed a proved relapse in two of those given pentaquine and none in those treated with pentaquine-quinine. About 90 per cent of his patients were followed and the clinical relapse rate in the two groups was 13.6 and 12.5 per cent, respectively. The relapse rates were significantly less than in similar patients given pamaquine, paludrine, pamaquine-paludrine, or pamaquine-quinine.

Coggeshall and Rice[15] treated 185 patients with pentaquine-quinine for naturally acquired vivax malaria. Most of them had the tropical-zone type with many previous relapses. Perhaps because of acquired immunity, it was possible to reduce the dose of pentaquine to 30 mg. daily and quinine to 1.0

TABLE III. RELAPSE OF VIVAX MALARIA AFTER PENTAQUINE ALONE AND IN COMBINATION (A REVIEW OF THE LITERATURE)

AUTHORS	YEAR	TYPE OF MALARIA	NO. PATIENTS	TREATMENT	% WITH PROVED RELAPSE
Alving et al.[4]	1948	Experimental tropical zone (Chesson)		(60 mg./day + quinine) × 14	
		Moderate	26		4
		Severe	17		18
		Massive	4		100
Coatney et al.[13]	1950	Experimental tropical zone (Chesson)	17	(60 mg./day + quinine) × 14	Variable (25-100)
Monk[14]	1948	Natural tropical zone	25	60 mg./day × 10	8
			26	Same + quinine	0
Coggeshall and Rice[15]	1949	Natural tropical zone	185	(30 mg./day + quinine) × 14	5.4
Straus and Gennis[16]	1950	Natural tropical zone	50	(60 mg./day + quinine) × 14	2
Eldin and Morcos[17]	1952	Natural tropical zone	22	(30 mg./day + quinine) × 14	4.5
			18	Same × 5	16
White et al.[18]	1948	Experimental temperate zone (St. Elizabeth)	15	(60 mg./day + quinine) × 14	0
Hall and Latts	1954	Natural temperate zone	35	(30 mg./day + quinine) × 14	0

Gm. daily for 14 days. Clinical control was satisfactory and no relapses occurred in 163 (88 per cent). Ten had proved relapses and 10 others had recurrent chills.

Straus and Gennis[16] carried out a controlled study with tropical-zone vivax malaria very similar to the present experiment. The follow-up period was one year or more in 72 per cent. Relapses occurred in 34.6 per cent of 49 patients given chloroquine and in only 2 per cent of 50 patients treated with pentaquine-quinine.

Eldin and Morcos[17] used 30 mg. of pentaquine and 0.9 Gm. of quinine daily for vivax malaria in Egypt. Twenty-two patients were treated for 14 days; follow-up study for 6 months disclosed one relapse. Eighteen patients were treated for only 5 days and 16 per cent had a relapse.

White and associates[18] used pentaquine-quinine successfully for the radical cure of experimental temperate zone vivax malaria in 15 subjects. Pentaquine was given in a dose of 60 mg. daily with quinine, 2.0 Gm. daily, for 14 days.

In the present study with naturally acquired, temperate-zone vivax malaria, chloroquine controlled fever and parasitemia somewhat more quickly than pentaquine-quinine. However, patients treated with chloroquine had recurrent symptoms of malaria four times more often than those given pentaquine-quinine. None of the patients given the latter therapy had a proved relapse. The number and frequency of relapses in both treatment series were less than with tropical-zone vivax malaria.

Recently Gennis and collaborators[19] gave primaquine, 15 mg. daily, for 14 days to 101 patients with Korean vivax malaria. Clinical response was good with normal temperature in an average of 2.1 days and negative blood smears in 4.3 days. Follow-up study for an average of 12.2 months showed

five relapses in 5 to 60 days. The incidence of recurrent chills was not reported. Drug toxicity was minimal, probably because of the small daily dose. A study of therapy with pentaquine in a dose of 15 mg. daily has not been reported. The advantage anticipated with this type of therapy is a high cure rate without toxicity and the necessity of hospital observation during treatment. Chloroquine or quinine should be added to reduce the duration of parasitemia and fever.

The potential toxicity of pentaquine has been proved and has led to its replacement by primaquine in the radical cure for vivax malaria.[19-21] The toxicity of pentaquine is directly related to the daily dose. Doses of 120 to 180 mg. daily produced severe abdominal pain, nausea, anorexia, cyanosis, and methemoglobinemia in most experimental subjects.[22] With 60 mg. daily, moderate epigastric pain, anorexia, and methemoglobinemia have been frequent.[4, 13, 16] Drug fever was an occasional side effect. Doses of 15 to 45 mg. daily have produced only a slight methemoglobinemia. The effect of 60 mg. pentaquine was equivalent to 30 mg. pamaquine daily. Concurrent administration of quinine did not increase the toxicity of pentaquine.* Acute hemolytic anemia rarely has followed the use of pentaquine and has done so more often in Negro patients.[4, 14]

Simultaneous administration of quinacrine (Atabrine) with pentaquine proved to be undesirable since the average concentration of methemoglobin was three times that seen with pentaquine and quinine.[23] Severe leukopenia has been observed when sulfonamides were given with pentaquine.[23]

Toxicity from pentaquine was surprisingly uncommon in the present study (Table I). There was one instance of drug fever and rash caused by pentaquine and no other striking side effect. Toxicity from quinine was also minimal. The low toxicity was undoubtedly the result of limiting the dose of pentaquine to 30 mg. daily. A similar low incidence of serious side effects with this dose has been noted by others.[4, 15, 17] In our experience 30 mg. of pentaquine daily has been no more toxic than an equal dose of primaquine.

SUMMARY AND CONCLUSIONS

A controlled study was carried out in 101 patients having temperate-zone vivax malaria contracted in Korea. Alternate cases were treated with chloroquine or pentaquine and quinine. Chloroquine controlled the fever and parasitemia a little more quickly than pentaquine and quinine. Toxic reactions were rare with either treatment. Proved relapses occurred in one-fourth of those given chloroquine and in none of those on pentaquine and quinine. Lasting cure of symptoms was seen in two-thirds of those given pentaquine and quinine and only 17 per cent of those given chloroquine. The best treatment of acute vivax malaria might be pentaquine or primaquine, 15 mg. daily, for 14 days with chloroquine, 1.0 Gm. the first day and 0.5 Gm. for the next three days.

*The administration of quinine with large doses of primaquine reduced abdominal pain, methemoglobinemia, and plasma primaquine concentration.[19]

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A PROTECTIVE EFFECT OF A CARBON-FREE FRACTION OF INDIA INK AGAINST SNAKE VENOM

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WITH THE TECHNICAL ASSISTANCE OF ANN WACKER

IN A footnote to a paper on spreading factor in certain snake venoms, Duran-Reynals[1] reported that when India ink was injected into the same area as snake venom, the lesions produced by the venom appeared smaller and less severe. This suggested to us that India ink, or one of its constituents, might be useful as a treatment for snakebite. Accordingly, the following experiments were performed on rats and mice, using both rattlesnake and cobra venom. Initially, the whole ink was used, and later, some of its separated components.

METHOD

Each batch of venom was tested to determine a dose which would be lethal for between 50 and 95 per cent of the animals. The control animals then received this dose subcutaneously, followed in 2 minutes by 2 ml. of saline solution injected into the same area. The treated animals were given the venom, followed in 2 minutes by 2 ml. of India ink or another test substance into the same area. Since the dissolved venom gradually loses potency, the control and treated animals were alternated, sometimes by groups of 1 or 2, and sometimes by cages of 6 animals each. The times of death, up to 96 hours, were recorded and graphed. Thus, it was possible to determine, not only the effects of the test substances on mortality rate, but also any prolongation of life. Initially, the Higgins brand of India ink was used. Comparisons were also made with another brand.

RESULTS

Table I shows the protective effect of Higgins India ink in four experiments on 124 rats. It is clear that the India ink reduced the total mortality by about one-half. In addition, there was a marked delay in the lethal effects of the cobra venom, because the 12-hour mortality was 72 per cent in the controls, and only 11 per cent in the treated.

In the next experiment another brand of India ink (Stafford's), tested in mice given rattlesnake venom, 8 mg. per kilogram, did not reduce and may have increased the mortality rate, compared to the controls. Since it appeared that only the Higgins brand of India ink afforded protection, tests were made of several of its known constituents. Suspensions of carbon black, of a size similar to that used in Higgins India ink, were tested on both rats and mice, and did not reduce the mortality rate from rattlesnake venom. On the other hand, the carbon-free fraction of Higgins India ink produced by high-speed

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TABLE I. THE PROTECTION BY INDIA INK AND SOME OF ITS CONSTITUENTS AGAINST SNAKE VENOMS IN RATS

PROTECTIVE SUBSTANCE	VENOM*	DOSE OF VENOM (MG./KG.)	NO. OF RATS USED		12-HR. MORTALITY (PER CENT)		96-HR. MORTALITY (PER CENT)	
			CONTROL	TREATED	CONTROL	TREATED	CONTROL	TREATED
Higgins India ink	Rattlesnake (C. adamanteus Lot 1	50	12	12	25	0	83	25
Higgins India ink	Rattlesnake Lot 1	25	12	12	0	0	42	8
Higgins India ink	Rattlesnake Lot 2	20	20	20	20	10	95	55
Higgins India ink	Cobra (N. naja)	0.4	18	18	72	11	89	56
Higgins India ink —carbon-free supernatant	Rattlesnake Lot 3	15	24	24	33	13	83	33
Redissolved precipitate from Higgins India ink supernatant	Rattlesnake Lot 3	18	21	21	No record	No record	71	33

*The potency of each lot of venom varied considerably, depending on many factors.

centrifugation gave a significant degree of protection (Table I). The pH of the Higgins carbon-free fraction was found to be 8.8 Upon acidification, a precipitate formed. This precipitate was filtered off, and redissolved at a pH of 8.8. When administered to rats, it, too, provided a significant degree of protection against rattlesnake venom (Table I).

The ink contains a mixture of many ingredients, and its exact composition is not known. Through the kindness of Dr. Samuelson of Columbian Carbon Company, a list of the major known ingredients was obtained; it was found to consist of ammonium hydroxide, complex organic acids, esters of shellac with ethyl alcohol, and compounds formed by boiling gelatin with ammonium hydroxide. Each of these substances was tested and failed to show any protection against rattlesnake venom. Several seemed to increase the fatality rate. Thus, the substance responsible for the protection against snake venom is as yet unidentified.

COMMENT

In the past it has been customary to ascribe the actions of India ink to the suspended carbon particles. This study clearly indicates that a substance in the supernatant fluid has important pharmacologic activity. Zilversmit, Shore, and Gantt[2, 3] have shown that the action of India ink on Bromosulfalein excretion is not due to reticuloendothelial blockade by the suspended carbon particles, as had been thought previously, but rather to the activity of some soluble substance in the aqueous phase. Since they also used the Higgins brand of India ink, it may be that the same component which is responsible for the inhibition of B.S.P. clearance also protects against snake venoms.

It is of interest that Favilli and McClean[4] found that a German brand of India ink counteracted an active principle in testicular extract. Although they attribute their results to the carbon particles in the ink, it is possible that here, too, a component of the carbon-free filtrate is involved.

Since Higgins India ink protects against both rattlesnake and cobra venoms, which are dissimilar in their mode of producing death, it seems likely that the protective effect would apply to other snake venoms as well. This suggests a possible therapeutic application. Although antivenins of great efficacy are available for the commoner poisonous snakes, they are not widely distributed. Frequently, there is a long delay before a victim can reach a source of antivenin. Since the bite of a large Florida diamond-back rattlesnake can be fatal within one-half hour,[5] and the venom of cobras and other tropical species is even more deadly, a readily available universal antidote would be of great value, even if it merely delayed the lethal effects of the venom until the victim could receive the specific antiserum. The importance of snakebite as a cause of death is indicated by the estimate that throughout the world 30,000 to 40,000 persons a year die of it.[6]

The protective effect observed in these experiments was only moderate. However, if the active ingredient in Higgins India ink could be administered without the other constituents of the mixture, some of which seem to increase mortality, it seems likely that a much greater protective effect would result. These experiments only show an effect of India ink injected subcutaneously into the same area as the venom. Studies on other routes of administration and on mechanism of action have been deferred until identification and isolation of the active material.

Finally, since substances similar to those found in snake venoms may be involved in infections and malignancies,[7, 8] the eventual solution of this problem may have important ramifications in other areas.

SUMMARY

In a series of experiments on rats and mice it was found that some as yet unidentified substance in the carbon-free fraction of Higgins India ink affords a significant degree of protection against the lethal effects of both rattlesnake and cobra venoms when injected subcutaneously into the same area as the venom. Another brand of India ink did not protect. A number of known constituents of India ink were tested and also found to be ineffective. Some of the implications of these findings are discussed.

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DISSOCIATION OF COAGULATION ABNORMALITIES IN IDIOPATHIC THROMBOCYTOPENIC PURPURA

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IDIOPATHIC thrombocytopenic purpura has long been known as a hemorrhagic disease of unknown cause, with marked platelet reduction and poor clot retraction as its characteristic blood findings.[1-3] Recently, however, the advent of new testing techniques has made possible further clarification of the coagulation disturbance in idiopathic thrombocytopenic purpura.

Using the prothrombin consumption test,[4] Soulier[5] and Quick and associates[6] were able to measure a defect in prothrombin utilization in this disease. Although the correlation between low platelet number and poor clot retraction had been known since Hayem's work in 1895,[7] development of the blood resistance method by Rosenthal and Tobias[8] and others[15] provided an objective means of determining onset and rate of clot retraction.

Many authors[3, 6] have concluded that prothrombin consumption and clot retraction are dependent on the quantity of platelets present in this disease. The following is a report of a patient with idiopathic thrombocytopenic purpura who showed the typical defects in platelet number, clot retraction, and prothrombin consumption. However, immediately following splenectomy there was a rapid improvement of prothrombin consumption, not paralleled by a rise in platelet count and clot retraction rate.

MATERIALS AND METHODS

Case Report.—The patient was a 22-year-old white man who noted the onset for the first time Aug. 15, 1953, of small petechial hemorrhages in his mouth, and multiple petechiae and fatigue of the legs. Gum bleeding and a prolonged nosebleed were observed also. A platelet count of 9,000 was made at that time. Continued petechiae and the development of purpura over the patient's body resulted in admission to the Randolph Air Force Base Hospital on Oct. 8, 1953.

The patient's family and occupational history did not aid in determination of the etiology of his illness. Physical findings included multiple petechiae and ecchymotic areas covering buccal mucosa, soft palate, abdomen, upper and lower extremities, and particularly dependent portions. The spleen was palpable three fingerbreadths below the left costal margin, firm, and nontender.

Pertinent laboratory data, other than coagulation studies reported hereinafter, revealed a hemoglobin of 16 Gm., a red cell count of 4,970,000 per c.mm., and a white cell count of 5,100 per c.mm. with a normal differential. Sternal marrow aspiration was normal except for atypical megakaryocytes. These were normal in number but showed marked diminution

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TABLE I

DATE (1953)	PLATELET COUNT[4]	BLEEDING TIME[4] (MIN.)	CLOTTING TIME[4] (MIN.)	CLOT RETRACT. RATE[8] $\left(\frac{\text{OHM-CM.}}{\text{MIN.}}\right)$	RUMPEL LEEDS[4] (1-4+)	PLASMA PROTHROM. TIME[24] (SEC.)	SERUM PROTHROM. TIME[9, 10] (SEC.)	PLATELET AGGLUTINATION[11] (1-4+)	REMARKS
Normal	150,000 to 500,000	Up to 3	5 to 10	5 to 8	0	13.5 to 15.5	Over 25	0 to +	
Oct. 8	9,900	3:30	4:00	0.00	++++	13.3	12.0		
Oct. 12	9,900	21:30		0.00		13.8	13.0	++	Cortisone therapy begun
Oct. 19	12,620			0.00	++++	15.7	15.0		
Oct. 22	9,500	30:00	9:00	0.00	++++	14.5	15.0		
Oct. 28	9,800	7:00		0.50	++	14.5	14.7		
Nov. 4	10,700	6:00		0.50	+	14.2	12.6		
Nov. 10	4,750	3:50	5:00	0.25		13.9	15.1	+	Preoperative
Nov. 10	4,930		3:00	0.25		14.4	25.1		5 min. } After
Nov. 10	14,780		3:30	0.38		14.5	33.4		30 min. } splenic pedi-
Nov. 10	20,570		4:00	0.13		14.8	28.1		60 min. } cle clamped
Nov. 11	502,860		5:00	3.75	0	15.9	39.0		
Nov. 13			5:00			15.2	180.0	+	
Nov. 19	470,000	1:30		1.90		14.5	111.4		
Nov. 20	526,000			5.90					
Nov. 23	273,000	1:50		5.90		14.9			Cortisone discontinued
Nov. 28	479,220		4:30	4.65	0	15.4	80.2	±	

of cytoplasmic granularity and no evidence of platelet formation. Platelets in marrow and peripheral smears were few in number, and a moderate proportion of them were larger than normal. Spleen sections were reported as "consistent with thrombocytopenic purpura."

The diagnosis of idiopathic thrombocytopenic purpura was made and, on Oct. 13, 1953, the patient was started on cortisone, 100 mg. every eight hours. This dosage was continued until the patient was operated upon on Nov. 10, 1953. During this time the patient developed definite signs of hypercortisonism, and the petechial hemorrhages and leg pain diminished in severity. On Nov. 9, 1953, the patient was prepared for surgery with cortisone acetate, 50 mg. intramuscularly every four hours. This was continued through the day of surgery and for three succeeding days. In addition, a 100 mg. tablet of cortisone was given, orally, one hour before the operation. The cortisone dosage was decreased in decrements of 25 mg. daily, and discontinued on Nov. 23, 1953, after 41 days of therapy. The splenectomy proceeded uneventfully and a grossly enlarged spleen was removed at the time of surgery. Two pints of blood, each 21 days old, were given beginning 90 minutes postoperatively. The postoperative course was uneventful.

METHODS

Anticoagulant.—Sequestrene (Alrose Chemical Company) was used in a 0.1 per cent dilution.

Venipunctures.—These were performed using syringes and needles coated internally by Silicone oil (GE #9996-300).

Platelet Count.—Platelet counts were performed on finger-tip and Sequestrene-venous blood by the direct method.[4]

Bleeding Time.—This test was made on an ear-lobe incision according to the method described by Quick.[4]

Clotting Time.—This test was performed by placing 1 ml. of blood into a 13 × 100 mm. test tube at 37° C. and tilting it every thirty seconds, as described by Quick,[4] until blood did not run on inversion of tube.

Clot Retraction Rate.—1.5 ml. of whole blood was placed in a platinum electrode resistance cell and incubated at 37° C. for 40 minutes according to the technique of Rosenthal and Tobias.[8] Continuous automatic recordings of resistance changes[15] were used in determining the rate of clot retraction.

Rumpel-Leeds Tourniquet Test.—This procedure was performed by occluding the blood of the upper extremity to a level midway between systolic and diastolic pressure for 5 minutes. The number of petechiae which occurred in the succeeding 15 minutes was recorded and graded according to relative quantity.[4]

Plasma Prothrombin Time.—This test was performed according to the Link-Shapiro modification[24] of Quick's one-stage procedure.[4] Simplastin (Warner-Chilcott) was the thromboplastin reagent used.

Serum Prothrombin Time.—This procedure was a modification[9, 10] of Quick's prothrombin consumption test.[4] The time required for serum from blood incubated 60 minutes to clot, following the addition of Simplastin and purified fibrinogen (Warner-Chilcott), was determined.

Platelet Agglutination Test.—The immunologic procedure described by Harrington and co-workers[11] was followed.

RESULTS

Data obtained from tests pertinent to the patient's hematologic disturbance are presented in Table I. It will be noted that there was an immediate and continued increase in prothrombin utilization following splenic vessel ligation. No real increase in platelet count or clot retraction rate occurred in the first hour after surgery. The decrease in the abnormality of the bleeding time, tourniquet, and platelet agglutination tests on cortisone therapy were maintained following splenectomy.

DISCUSSION

The commonly accepted concept that both clot retraction and prothrombin utilization abnormalities in this disease stem from the low number of platelets[3, 6] does not explain the immediate improvement in prothrombin consumption before any real change in the other hematologic abnormalities following splenectomy. It is interesting to note that in the only study found in a review of the pertinent literature on the effect of splenectomy on idiopathic thrombocytopenic purpura utilizing similar hematologic techniques in the same observation period, similar findings resulted. Quick's data[6] reveal the same trend, although not as marked, toward dissociation of rate of platelet increase and prothrombin consumption recovery following splenic vessel ligation. He suggested that it could perhaps be explained by platelet counting errors due to the technical difficulties of blood specimen collection during surgery.

Stefanini, Damashek, and colleagues,[20] studying the in vivo effect of platelet transfusions in a patient with idiopathic thrombocytopenic purpura and a strong platelet agglutinin, noted that although the platelet count was below base line by the end of the transfusions and took two hours to return to base line, the utilization of prothrombin during clotting temporarily became normal and took four hours to return to original values.

A discrepancy between platelet count and prothrombin consumption in quinidine-induced thrombocytopenia was pointed out by Hirsch and Damashek.[32]

Experimentally, lack of correlation in rate of recovery of prothrombin consumption and platelets has been observed following radiation injury. Cronkite[12] found that following whole-body irradiation, prothrombin utilization and platelet count decreased in parallel fashion. In the recovery phase, however, utilization reached 100 per cent with platelets still at a level which, in the destructive phase, was accompanied by only 10 per cent utilization. Similar findings have been observed in monkeys,[13] rabbits,[13] burros,[14] and dogs.[19]

Quick, Shaneberg, and Stefanini[22] showed that, all other components being normal, the speed of prothrombin conversion was a function of the number of platelets present. In our case and all the instances discussed previously, however, the primary defect, i.e., the thrombocytopenia, was not apparently corrected significantly when prothrombin utilization improved. It is possible that the evidence and suggestion of Doan[23] are pertinent that the peripheral blood platelet count may represent the surplus and, therefore, not be a true criterion of thrombocytopenia in periods of high platelet consumption. That the low platelet counts are all due to technical error is less likely. However, even if the possibility is accepted that the actual functional level of thrombocytes is higher than indicated by count, the discrepancy between prompt prothrombin consumption increase and delayed clot retraction improvement in our case still requires elucidation.

A possible explanation of the dissociation has been suggested by the division of platelet activity by Quick[4] into two primary functions, i.e., thromboplastinogen activation, and vasoconstrictor liberation, and by Stefanini[25] into clot retraction, degree of capillary fragility, bleeding time, and prothrombin utiliza-

tion during clotting. Pertinent clinical instances have been reported of a hemorrhagic syndrome in the presence of a normal platelet count with (a) delayed clot retraction, prolonged bleeding time, and normal prothrombin consumption,[26] (b) normal clot retraction, normal bleeding time, and deficient prothrombin consumption,[21, 27] (c) delayed clot retraction and decreased prothrombin consumption,[28] and following splenectomy, of normal platelet number, but continued high titer of platelet agglutinin and increased bleeding time.[20] In our case, there may have been an abrupt cessation of an inhibitory action of the spleen on the thromboplastinogen-activation function of the platelets immediately following splenic vessel ligation. On the other hand, the greater delay in improvement of the clot retraction function may have been the result of the longer time required for the postsplenectomy production and circulation of thrombocytes no longer deficient in this function.

The possibility also exists that the presence of a platelet agglutinin played a part in producing the discrepancy noted herein. The platelet agglutination test of Harrington[11] was moderately positive but showed some improvement on cortisone and splenectomy therapy. Stefanini[20] noted a discrepancy in findings in a case of idiopathic thrombocytopenic purpura in which the agglutinated platelets showed decreased clot retraction power but increased prothrombin utilization. He postulated that the agglutinin was able to injure the platelets in sufficient degree to inhibit clot retraction, simultaneously liberating or activating certain factors which influence the rate and extent of prothrombin utilization during clotting. In our case, however, this postulate would not explain why the clot retraction inhibition effect should have been present at a time when the prothrombin utilization was decreased.

If the low peripheral platelet counts in the cases cited are truly indicative of thrombocytopenia, the observed increases in prothrombin consumption are not easily explained. Stefanini and colleagues[20] demonstrated temporarily normal prothrombin utilization in a thrombocytopenic patient during a period following platelet transfusion in which platelet counts were even lower than baseline. Cronkite and colleagues[12] noted that the in vitro rate of prothrombin conversion of postradiation thrombopenic canine blood on addition of very low concentrations of platelets (e.g., 8,000 per c. mm.) was much more rapid than that seen in normal freshly shed blood. The possible presence of a plasma substance in thrombocytopenic blood, particularly in the recovery phase, which in some way increases thromboplastinogen activation without additional platelets warrants consideration.

This case seems to give additional support to the concept of the existence of a number of possible platelet dysfunctions, and adds the further possibility that the mechanism which brings about recovery from one dysfunction may not necessarily correct another one in the same way or time—if at all. It also demonstrates, in common with the other cases discussed above, that the relationships between platelet number and various clotting functions are not as direct and clear as perhaps has been assumed. Further study is indicated to clarify these relationships.

It should be noted that, in this case, cortisone was used for a month preoperatively to decrease the capillary hyperpermeability seen in this disease.[17, 18, 29-31] It was hoped that this would protect the patient from any serious hemorrhagic phenomena while awaiting a spontaneous remission. The clinical improvement and the decrease in bleeding time, in positive response to the tourniquet test and, perhaps, in platelet agglutination, indicate some value in this procedure. Cortisone therapy was continued through the operative and immediate postoperative period for the same reason, with beneficial effects on hemostasis according to the surgical staff.

SUMMARY

A return toward normal prothrombin utilization prior to platelet and clot retraction recovery is observed in a case of idiopathic thrombocytopenic purpura following splenectomy. The occurrence of this recovery pattern in other reported cases of thrombocytopenic purpura and following experimental whole-body irradiation is pointed out. The concept of multiple separate platelet dysfunctions and their mechanisms is considered, as is the relationship between platelet quantity and blood coagulation. The efficacy of cortisone therapy in reducing the capillary permeability of idiopathic thrombocytopenic purpura is suggested.

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SERUM CONCENTRATIONS OF VITAMIN B_{12} IN NORMAL AND NUTRITIONALLY DEFICIENT MONKEYS

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VITAMIN B_{12} is apparently an essential and critical factor in the metabolism of most body cells, particularly those concerned with hematopoiesis. The precise role of this substance in protein synthesis and its relationship to other essential vitamins, especially to pteroylglutamic acid and ascorbic acid, are not yet clear. This relationship, however, is of such apparent importance that we have been interested in determining vitamin B_{12} serum concentrations during an investigation of the role of vitamin B_{12} and other nutritional factors in hematopoiesis of rhesus monkeys.

METHODS

Serum concentrations of vitamin B_{12} have been assayed serially in apparently normal *M. mulatta* monkeys on "natural" diets and on various experimental diets. These dietary regimes were as follows:

(a) "Natural" laboratory diets consisting of Rockland monkey feed, carrots, lettuce, sunflower seeds, and homogenized pasteurized whole cow's milk. A sample of this milk was assayed to contain 3.24 μg of vitamin B_{12} per liter.

(b) "Vitamin-free" basic diet supplemented with measured oral daily doses of vitamins. The basic diet consisted of sucrose 72 per cent, vitamin-free casein 18 per cent, vegetable oil 4 per cent, salt mixture (USP 2) 4 per cent, and cod liver oil 2 per cent. Assay of this basic diet showed it to contain 0.005 μg of vitamin B_{12} per gram. The standard daily vitamin supplement consisted of thiamine hydrochloride 1 mg., pyridoxine hydrochloride 1 mg., calcium panthothenate 3 mg., niacin 5 mg., choline chloride 25 mg., para-aminobenzoic acid 50 mg., inositol 50 mg., folic acid 0.1 mg., riboflavin 1 mg., and ascorbic acid 25 mg.* The ascorbic acid and folic acid were omitted in certain regimes and the vitamin B_{12} supplement was varied to suit the experimental purposes, as will be noted subsequently.

(c) Certain animals on dietary Regime 2 had previously been maintained for long periods of time on homogenized whole cow's milk rendered ascorbic acid-free by treatment overnight with copper sulfate and subsequent boiling. A sample of this treated milk was assayed and found to contain 2.72 μg per liter of vitamin B_{12}.

Serum concentrations of vitamin B_{12} were determined microbiologically using *Euglena gracilis* as test organism,[1] and the technique described by Ross[2] for human serum assay. In human serum the vitamin exists as a labile complex with serum protein and is, in the natural state, unavailable to the Euglena. When human serum is assayed without preliminary heating, there is usually very little growth. Any growth which does occur is considered due to "free" or "easily available" vitamin, B_{12}. Growth is much more active if the

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serum is heat denatured before its vitamin B_{12} content is assayed. The difference in growth between the heated and the unheated preparations represents the concentration of vitamin B_{12} in the "unavailable" or "bound" form.

Euglena gracilis is stimulated to demonstrable growth by the addition to the basal medium of as little as 1$\mu\mu$g (micromicrogram) of vitamin B_{12} per milliliter. However, for technical reasons, it is not possible to assay serum concentration of vitamin B_{12} below 16 $\mu\mu$g per milliliter by the method. In this paper, serum concentrations less than 16 $\mu\mu$g per milliliter are recorded as zero concentrations.

In several instances the vitamin B_{12} activity of monkey feces was determined. An aliquot of fresh feces was weighed and then homogenized in a known volume of phosphate buffer, pH 7.5. The mixture was autoclaved and filtered, and the clear filtrate assayed for its vitamin B_{12} activity by the *Euglena gracilis* method. Values were expressed as micrograms per gram of fresh feces.

RESULTS

A. *Comparison of the Normal Concentrations in Human and in Monkey Sera.*—In 56 normal human sera a range of total concentration of vitamin B_{12} from 86 to 460 $\mu\mu$g per milliliter was found, with a mean of 212 $\mu\mu$g per milliliter. In 52 of the sera the vitamin B_{12} was estimated to be wholly in the "bound" or "unavailable" form. In the other 4, small amounts of "free" vitamin were found. The mean "bound" concentration for the whole group was 207 $\mu\mu$g per milliliter.

TABLE I. SERUM VITAMIN B_{12} LEVELS ON ANIMALS WHICH HAVE BEEN ON NORMAL DIETS FOR SEVERAL MONTHS

MONKEY NUMBER	TOTAL B_{12} ($\mu\mu$G/ML.)	"EASILY AVAILABLE" B_{12} ($\mu\mu$G/ML.)	PER CENT
D4	234	Not obtained	--
D3	200	72	36.0
H11	190	116	65.0
20	152	128	84.2
D2	146	132	90.4
48	144	54	37.5
114	136	--	--
15	120	58	48.3
115	106	--	--
8	100	56	56.0
78	100	52	52.0
H1	88	56	63.7
19	84	46	44.5
D1	70	40	57.2
H14	70	40	57.2
D5	70	--	--
H15	50	33	66.0

Table I lists the serum vitamin B_{12} concentrations found in 17 young monkeys which had been on natural diets ad libitum for several months. The range of total vitamin B_{12} concentration varied from 50 to 234 $\mu\mu$g per milliliter with a mean of 120 $\mu\mu$g per milliliter. All but one showed a total concentration of 70 $\mu\mu$g per milliliter or more. Both the range and the mean value are somewhat lower than are found with human sera. In 13 of these animals, concentrations of "free" or "easily available" vitamin were determined. In all samples tested, appreciable amounts of this form of the vitamin were found, and in 11 of the 13 a value greater than 40 $\mu\mu$g per milliliter was observed. The range of "easily available" vitamin in these 13 sera was from

33 to 132 μμg per milliliter, with a mean value of 69 μμg per milliliter. Thus, more than 50 per cent of the mean total vitamin B_{12} content of these sera was readily available to the Euglena. This is in contrast to human serum in which, as stated previously, it is unusual to find more than a trace of the free vitamin.

In two animals on natural diets (78 and 114), serum vitamin B_{12} concentrations were assayed on two occasions to determine the fluctuations in values which could be expected in the same animal. In Monkey 78, total values of 100 and 196 μμg per milliliter were obtained 124 days apart. In Monkey 114, values of 136 and 156 μμg per milliliter were obtained 14 days apart.

B. *Observations on Animals Maintained on Basic Diets With Varying Folic Acid and Vitamin B_{12} Supplements.*—Four monkeys (108, 110, 111, and 113) were maintained on vitamin-free basic diet (Regime 2) supplemented with the standard vitamin supplements including 1 μg of vitamin B_{12} per day but excluding folic acid. All of the animals developed serum concentrations of vitamin B_{12} at or below the lower limits of normal after they had been on the diet for periods varying from 130 to 242 days. Three of the four showed total vitamin B_{12} serum levels of 25 to 54 μμg per milliliter. One monkey (111) was treated with Aureomycin, 50 mg. daily, for 33 days of the 166 days of the dietary regime. The serum level of this animal was zero shortly after cessation of the Aureomycin but gradually rose to 54 μμg per milliliter thereafter.

The same four animals were studied further on a similar diet with the addition of folic acid in the standard daily dose and an increase of vitamin B_{12} to 2 μg daily. Slight, if any, change in the vitamin B_{12} serum levels was effected by this regime. Under these conditions total vitamin B_{12} values ranged from 40 to 68 μμg per milliliter in these four animals. In sharp contrast, however, the addition of the folic acid supplement *in the presence of frank folic acid deficiency* had a dramatic effect on the serum vitamin B_{12} levels (vide infra).

The oral vitamin B_{21} supplement was increased from 2 to 10 μg daily in Monkeys 108 and 110. Over a period of several weeks the serum levels rose from 44 to 250 μμg per milliliter and from 32 to 214 μμg per milliliter, respectively. An increase of the daily supplement to 20 μg in these two animals resulted in a further elevation of the serum concentration to 430 and 330 μμg per milliliter, respectively. After reduction of the oral supplement to 2 μg the concentrations returned to normal. The variations in serum concentration in these two animals are shown in Fig. 1.

C. *Serum Concentrations After Parenteral Administration of Vitamin B_{12}.*—Three of the animals referred to previously (110, 111, 113) were given 5 μg of vitamin B_{12} intravenously (Fig. 2). Forty-eight hours later the total vitamin B_{12} serum levels had risen from 48 to 230 μμg per milliliter, from 54 to 178 μμg per milliliter, and from 94 to 448 μμg per milliliter, respectively. When these animals were again tested 14 days later the serum concentrations in two of them had already fallen to a value just above the lower limit of normal; the serum concentration of Monkey 110 was assayed at 50 μμg per milli-

liter and of Monkey 111 at 64 $\mu\mu$g per milliliter. In Monkey 113, whose serum concentration rose the highest following the injection, a value of 138 $\mu\mu$g per milliliter was obtained 14 days later.

D. *Effect of Milk Diet on Serum Vitamin B_{12} Levels.*—Four animals (99, 100, 101, and 102) which had previously been maintained on ascorbic acid-free milk diets for periods of from 413 to 466 days were studied while on the basic diet with supplements deficient in folic acid but containing 2μg daily of vitamin B_{12}. This was a parallel study to that already described under heading "B" of Results. In the period of developing depletion during which classical signs of folic acid deficiency appeared, the range of vitamin B_{12}

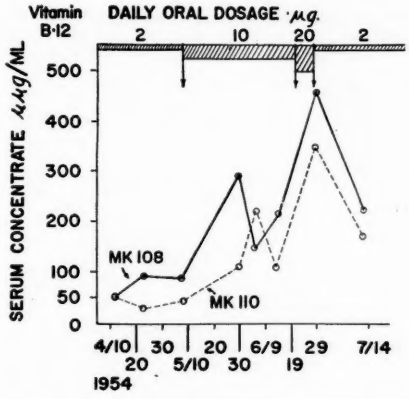

Fig. 1.—Serum concentrations of vitamin B_{12} with varying oral dosage of the vitamin.

serum levels was from 108 to 330 $\mu\mu$g per milliliter, 100 to 280 $\mu\mu$g per milliliter, 54 to 214 $\mu\mu$g per milliliter, and 68 to 204 $\mu\mu$g per milliliter, respectively. Thus, the mean values were substantially higher following a prolonged period of milk feeding than were the levels in those animals which received basic diets following long periods on "natural" diet, despite the fact that the former were subjected to ascorbic acid and folic acid deficiencies for the substantial period of milk feeding.

E. *Folic Acid Deficiency.*—Classical physical and hematological signs of folic acid deficiency were observed in the presence of normal or high normal vitamin B_{12} serum levels.[4, 5] In Monkeys 100, 102, 108, and 113 the serum levels were 100, 103, 54, and 138 $\mu\mu$g per milliliter, respectively, when these animals were exhibiting signs of profound folic acid deficiency. However, the process of recovery from this deficiency, induced by the addition of folic acid to the basic supplements, was associated with a rise in the vitamin B_{12} serum levels in 2 of 3 animals. During this period the serum level in Monkey 102 rose from 103 to 215 $\mu\mu$g per milliliter and in Monkey 108, from 54 to 178 $\mu\mu$g per milliliter, while in Monkey 113 there was no significant change from 138 to 142 $\mu\mu$g per milliliter. In all cases serum concentrations declined after the period of recovery. A similar phenomenon was observed during recovery from scurvy (vide infra).

F. *Ascorbic Acid Deficiency.*—Vitamin B_{12} serum levels of four animals were studied in the presence of ascorbic acid deficiency. Two of these (101 and 102) were on ascorbic acid-deficient whole milk supplemented with standard supplements including vitamin B_{12}, 2 mg., and lacking only ascorbic acid. Obvious signs of scurvy developed in these animals in 120 and 115 diet days, at which time the serum vitamin B_{12} levels were 67 and 20 $\mu\mu$g per milliliter, respectively. Both animals recovered rapidly in response to the parenteral administration of 100 mg. of sodium ascorbate every other day for four days followed by oral ascorbic acid, 25 mg. daily. During the period of recovery from scurvy the vitamin B_{12} levels rose sharply. Thirty-five days after the beginning of ascorbic acid therapy the serum vitamin B_{12} level of Monkey 101 had risen from 67 to 140 $\mu\mu$g per milliliter, while that of Monkey 102 had risen from 20 to 154 $\mu\mu$g per milliliter in 38 days. The concentrations remained normal thereafter. Two other animals (114 and 115) were fed basic diet plus the standard daily supplement including vitamin B_{12}, 2 μg, and lacking only ascorbic acid. Monkey 114 developed frank scurvy in 70 days, during which time the serum

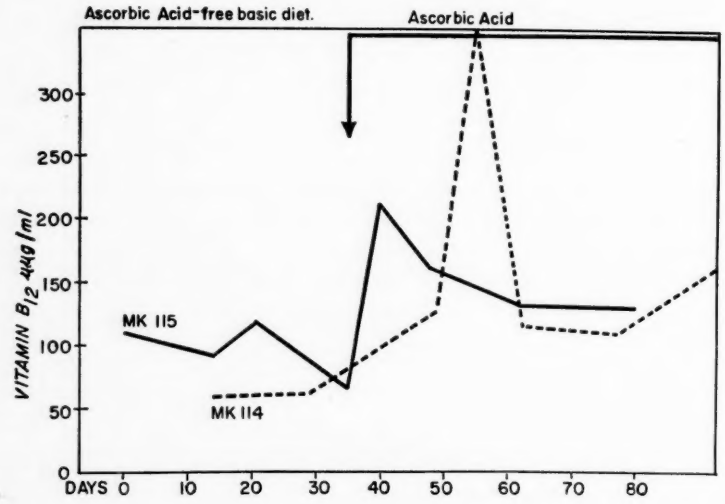

Fig. 2.—Response of serum vitamin B_{12} concentration to administration of parenteral ascorbic acid in scorbutic monkeys.

level of vitamin B_{12} fell progressively from 156 to 78 $\mu\mu$g per milliliter. Thirteen days after parenteral sodium ascorbate therapy was begun (50 mg. every second day for 16 days) the vitamin B_{12} serum level had risen from 78 to 126 $\mu\mu$g per milliliter, and to 664 $\mu\mu$g per milliliter nineteen days after therapy was started. Subsequent levels were within the normal range. Monkey 115 developed scurvy in 103 days, during which time the serum vitamin B_{12} levels varied rather erratically but progressed generally downward from a baseline level of 106 to 54 $\mu\mu$g per milliliter. Six days after parenteral sodium ascorbate was started (100 mg. every second day) the serum vitamin B_{12} level rose to 214 $\mu\mu$g per milliliter. It has since remained in the normal range.

During the 103 days of the experiment Animal 115 was used to test the vitamin B_{12}-retaining power of serum in the presence of this deficiency in the

same manner as that described previously in Monkeys 110, 111, and 113. Accordingly, 5 μg of vitamin B_{12} was given intravenously 28 days after the experimental diet was begun. Forty-eight hours after infection the serum vitamin B_{12} level had risen from 92 to 312 $\mu\mu$g per milliliter, fourteen days later had fallen to 132 $\mu\mu$g per milliliter, and ten days thereafter to 68 $\mu\mu$g per milliliter.

G. *Fecal Assays and the Relationship Between Fecal and Blood Vitamin B_{12} Concentrations.*—Because of the tendency of monkeys to develop diarrhea, especially in the presence of nutritional deficiency, it is difficult to follow critically the vitamin B_{12} excretion. However, a number of fecal and serum specimens were obtained simultaneously. The results are shown in Table II.

TABLE II. THE RELATIONSHIP BETWEEN FECAL AND SERUM VITAMIN B_{12} ASSAYS OBTAINED SIMULTANEOUSLY

MONKEY NUMBER	SERUM VITAMIN B_{12} ($\mu\mu$G/ML.)	FECAL VITAMIN B_{12} (μG/GM.)
100	192	0.62
101	140	0.14
102	154	0.45
108	178	0.15
	80	0.165
110	48	0.115
	25	0.20

It is evident that there is a wide range of variation in fecal vitamin B_{12} activity and that high fecal levels may be present when serum levels are extremely low. Conversely, similar serum levels may occur when there is a fourfold variation in fecal vitamin B_{12} activity.

SUMMARY

The vitamin B_{12} serum concentrations of *M. mulatta* monkeys were studied under a variety of dietary regimes and nutritional states. Under conditions of optimal nutrition the total vitamin B_{12} values ranged from 50 to 234 $\mu\mu$g per milliliter with a mean of 120 $\mu\mu$g per milliliter, while the "easily available" component ranged from 33 to 132 $\mu\mu$g per milliliter, with a mean value of 69 $\mu\mu$g per milliliter. These concentrations are in contrast to the human being in which there is a normal range of 86 to 460 $\mu\mu$g per milliliter, with a mean of 212 $\mu\mu$g per milliliter, most or all of which is in the "bound" form.

Monkeys maintained on synthetic folic acid-deficient diets with protein limited to 18 per cent casein and vitamin B_{12} limited to 1 μg daily showed a gradual decline of serum vitamin B_{12} levels to a range of 25 to 54 $\mu\mu$g per milliliter. The presence of the standard supplement of folic acid and an increase of vitamin B_{12} to 2 μg daily effected a slight, if any, increase in the serum vitamin B_{12} range. However, increasing the vitamin B_{12} supplement to 10 μg daily in two animals resulted in a prompt rise to high normal values, and 20 μg daily resulted in a further marked increase in serum levels. Intravenous administration of 5 μg of vitamin B_{12} to three of these animals which were moderately depleted and one animal on synthetic ascorbic-deficient diet resulted in high serum levels 48 hours after injection.

Animals which had been maintained for long periods of time on homogenized milk diets containing appreciable amounts of vitamin B_{12} showed higher serum levels when on synthetic diets than did the other monkeys which had been on "natural" diets before being put on synthetic diets. The only exceptions to this were the two animals which developed frank scurvy on milk diet (vide infra).

Normal vitamin B_{12} serum levels were observed in monkeys showing signs of severe folic acid deficiency. Under these conditions, however, recovery from the deficiency was accompanied by an increase in serum B_{12} levels in two of three animals.

Ascorbic acid deficiency was associated with low serum vitamin B_{12} levels in animals on milk diets and in those on synthetic diet. During the period of recovery from scurvy both groups of monkeys attained high serum vitamin B_{12} levels.

Simultaneous serum and fecal vitamin B_{12} assays indicated that substantial amounts of vitamin B_{12} are excreted in the presence of low as well as high serum vitamin B_{12} levels.

DISCUSSION

The mechanism of transport of vitamin B_{12} in monkey serum appears to differ from that in human serum. Total concentrations are lower and a considerable amount of the vitamin B_{12} in monkey serum exists in the "free" form. In human serum, vitamin B_{12} is bound to a protein which has an electrophoretic mobility of alpha globulin.[3] This protein is usually capable of holding in bound form the vitamin B_{12} naturally present in the serum. In addition, it is capable of binding limited amounts of vitamin B_{12} added in vitro. It has been thought that one effect of serum binding is to prevent loss of the vitamin by renal excretion. Free vitamin is found in human serum a short time after an injection of vitamin B_{12}, during which there is a considerable urinary excretion of the vitamin. The presence of a considerable amount of free vitamin in monkey serum suggests that a different mechanism operates in this animal.

The presence of high serum concentrations of vitamin B_{12} in Monkeys 110, 111, 113, and 115 forty-eight hours after an injection of 5 μg of vitamin B_{12} shows that monkey serum is capable of retaining in the circulation some part of the injected dose. Whether all or any of the vitamin is attached to serum protein is not yet known. Electrophoretic experiments on monkey serum have to date been inconclusive. The falling serum concentrations of those animals on a deficient diet, but with added supplements of vitamin B_{12}, is better explained by deficient intake associated with defective absorption from the gut than by faulty retentive capacity of the serum.

The daily vitamin B_{12} requirements and the limits of the range of serum B_{12} concentrations of the *M. mulatta* monkey are not known, but it was surprising to find that 2 μg vitamin B_{12} supplement daily was not sufficient to restore the low serum concentrations to normal in Animals 108, 110, 111, and 113. At

this time their diets were fully supplemented with folic acid and ascorbic acid. There was a rapid increase in serum concentrations to normal in two of these animals when the oral vitamin B_{12} daily supplement was increased to 10 μg. Because of the difficulties of collecting urine completely free of fecal contamination, we were unable to determine how much vitamin B_{12} is lost in the urine each day.

These studies suggest that the monkey is able to store considerable amounts of vitamin B_{12} as a reserve against depletion. Animals 99, 100, 101, and 102 had been maintained for a long period on an ascorbic acid-free whole milk diet which contained, however, considerable amounts of vitamin B_{12}. When subjected to deficiency states, their serum vitamin B_{12} levels remained higher than a similar group which had previously been on "natural" diet. On the other hand, however, the serum concentration in two animals reverted to the lower limit of the normal range 16 days after an intravenous injection of 5 μg of the vitamin.

The association of a low serum vitamin B_{12} concentration with the excretion of considerable amounts of vitamin B_{12} activity in the feces is not unexpected. Biosynthesis of vitamin B_{12} probably takes place in the large bowel of the monkey as it does in man. It is likely that there is a limited area of the gastrointestinal tract through which absorption of the vitamin B_{12} takes place, and that the synthesized vitamin is too far removed from the absorbing surface. In addition, faulty absorption was undoubtedly present in some of these deficient animals which had diarrhea. It is also known that feces contain variable quantities of a material which has vitamin B_{12} activity as judged by *Euglena gracilis* assay, but which is not hematopoietically active.

These data strongly suggest that in the monkey folic acid deficiency and ascorbic acid deficiency may both interfere with vitamin B_{12} absorption. Serum B_{12} concentrations have been reported in the normal range in the scorbutic human being.[9] The repeated observations of low vitamin B_{12} serum concentrations in scurvy in the monkey and the rapid rise of the serum levels during the recovery from scurvy in the presence of a constant daily dosage of the vitamin leave little doubt of the importance of ascorbic acid in vitamin B_{12} absorption. May and co-workers[6] assayed liver biopsies of scorbutic monkeys for vitamin B_{12} and folic and folinic acids. They pointed out that the folic and folinic acid content of the livers of scorbutic animals was much more reduced than was the vitamin B_{12} content. However, their data indicate that the vitamin B_{12} content was also significantly reduced. Furthermore, the addition of ascorbic acid resulted in a rapid increase in liver stores of folic and folinic acids as well as vitamin B_{12}. Their data seem to be consistent with the results of blood serum levels reported here and suggest that ascorbic acid may be a critical factor in influencing the absorption of folic acid as well as vitamin B_{12} and other nutritional factors.

The presence of normal concentrations in frank folic acid deficiency reaffirms previous observations of ourselves and others[7] that folic acid deficiency is unrelieved by the presence of abundant quantities of vitamin B_{12}. However,

in our experience the presence of 1 μg of vitamin B_{12} in the daily supplement postpones for some weeks the state of depletion which is considered characteristic of folic acid deficiency. The increased absorption of vitamin B_{12} during the recovery phase from folic acid deficiency in the monkey is similar to that observed in the absorption of iron[8] and probably many other nutrient factors.

The possibility that interference in ascorbic acid-folic acid metabolism may increase vitamin B_{12} utilization through some indirect mechanism is not borne out by the observations in folic acid deficiency.

CONCLUSIONS

The range of normal vitamin B_{12} levels in the blood of 17 *M. mulatta* monkeys has been determined. During prolonged periods on synthetic diets of minimal protein content, as outlined, monkeys develop low serum vitamin B_{12} levels which probably reflect depletion of body stores as well as inadequate supply or absorption. Under conditions of marginal diet, as outlined, it is shown that animals require more than 2 μg and probably less than 10 μg of vitamin B_{12} daily.

Normal serum vitamin B_{12} levels were observed in severe folic acid deficiency. However, recovery from this deficiency was associated with some increase in the serum level in two of three animals. This may reflect a temporary increase in absorption. Ascorbic acid deficiency resulted in marked interference with vitamin B_{12} absorption. Conversely, recovery from scurvy apparently accelerated vitamin B_{12} absorption. Animals which had previously been fed milk diets for long periods maintained relatively high serum vitamin B_{12} levels when placed on synthetic diets. This probably indicates a considerable capacity for vitamin B_{12} storage in the tissues of such animals.

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STUDIES IN IRON TRANSPORTATION AND METABOLISM

IX. The Excretion of Iron as Measured by the Isotope Technique

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ONE of the most distinctive characteristics of iron metabolism is the conservation and limited excretion of the metal by the body. Investigators who have studied the pathogenesis of iron-deficiency anemia have been so impressed with the efficiency of this conservation that they have sometimes implied that no iron is lost unless bleeding occurs. That such a concept is erroneous is evident from the fact that all cells contain some iron and cells are constantly being desquamated from the skin or mucous membranes. Iron, however, is so ubiquitous that it has been difficult, in ordinary balance experiments, to eliminate contamination and to differentiate unabsorbed from excreted iron in feces. Because these difficulties can be avoided by using radioactive iron, we have restudied the problem by giving isotopic iron intravenously. Evidence has been accumulated to indicate that small amounts of iron are regularly lost in sweat and from the gastrointestinal tract. It has not been possible to determine whether this loss represents true excretion or is primarily the result of cell desquamation. Calculation of total iron loss from the data for radioiron has also been difficult, but figures will be reported to indicate that the amount probably varies in man from 0.5 to 1.0 mg. per day.

Radioiron has been used by a number of investigators to study iron excretion in animals. In 1939, Hahn, Bale, Hettig, Kamen, and Whipple[1] injected radioactive iron into five dogs and followed excretion of the isotope in urine and feces for 7 to 88 days. They noted an output of 0.05 to 0.4 mg. per day. In two experiments red cell destruction induced by phenylhydrazine caused only a slight increase in iron excretion (0.1 to 1.0 mg. per day), the increase presumably coming from a greater amount of iron in bile. Copp and Greenberg[2] observed that rats, given Fe^{55} intravenously and sacrificed at 12, 24, 48, and 96 hours, showed a maximum excretion of 0.4 per cent of the dose. In another experiment in which Fe^{55} (half-life 2.9 years) was administered parenterally to small animals, Stevens, White, Hegsted, and Finch[3] determined the radioactivity in the total carcass of mice sacrificed at intervals over a period of 300 days. They found that one-half of the injected isotope had been lost by approximately 140 days. In no other species has a comparably high rate of iron excretion been observed.

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Much interest was aroused by the report of Mitchell and Hamilton[4] in 1949 that significant amounts of iron are lost through the skin. These workers induced sweating in three individuals, chemically determined the iron in sweat, and calculated that dermal loss of iron by the human subject, even under conditions of minimal sweating, might be as much as 6.5 mg. per day. Subsequent investigations have not confirmed these results.[5-7]

PLAN OF THE INVESTIGATION

We decided to re-explore the problem of iron excretion for two reasons. First, when tracer amounts of radioactive iron are given parenterally to normal subjects, a large portion of the injected isotope appears promptly in the circulating blood as newly synthesized hemoglobin.[8-10] If this radioiron were to be excreted, the excretion might be expected to be greatest at the time when the cells containing the tagged hemoglobin were being destroyed in large numbers—i.e., at about 100 to 120 days after the injection. Measurements of the radioiron excretion have been continued, therefore, for 140 days. Second, very few of the recorded studies of iron excretion have been made on subjects with hypochromic or iron-deficiency anemia. We wished to find out whether such individuals might have developed their deficiency because of a greater than normal excretion of the metal.

The plan of study was (1) to measure the fecal excretion of radioactive iron in five-day periods at intervals throughout 140 days following a dose of radioactive iron given intravenously or by mouth to normal individuals, to patients with hypochromic anemia, and to patients with hemolytic anemia; (2) to make similar studies in dogs having a hemolytic anemia induced by phenylhydrazine; (3) to measure the excretion of radioactive iron in sweat after injection of the isotope.

MATERIAL AND METHODS

Two different isotope preparations were used: (1) Fe^{59}, with a half-life of 46 days, prepared in the Washington University cyclotron by deuteron bombardment of cobalt and purified by extraction with isopropyl ether; (2) Fe^{59} accompanied by the three-year isotope, Fe^{55}, prepared from iron by neutron reaction in the pile at Oak Ridge and obtained through the Isotopes Division of the Atomic Energy Commission. The iron was synthesized into ferrous ascorbate for intravenous use.[8]

Normal Group.—Three men and one woman on the laboratory staff, all in good health, received 6 to 18 μc of Fe^{59} intravenously. Observations were also made on a student who had received 100 μc orally and had utilized 10 per cent of the tracer iron for hemoglobin formation.

Iron-Deficient Group.—Two women with hypochromic anemia, one seven months pregnant and another post partum, received radioiron intravenously. A third iron-deficient woman had taken the isotope orally three weeks before the beginning of observations.

Hemolytic Anemia Group.—A young woman with sickle cell anemia and a young man with hereditary spherocytosis were studied.

With the two exceptions mentioned, the isotope was given intravenously as ferrous ascorbate in amounts varying from 5 to 94 mg. of iron and from 4 to 20 μc. Quantitative collections of feces were made for five-day periods twice a month for 140 days and the radioactivity was measured by counting the β rays of the Fe^{59} with a Geiger counter. Each day's specimen was weighed and (in all experiments except those on the normal

subjects) was routinely tested for occult blood. The radioiron in each day's specimen was determined and the values for five consecutive days were averaged. An aliquot of a suspension of the 24-hour feces in 0.6 N HCl was wet ashed with concentrated sulfuric and perchloric acids. Before digestion, 2 mg. of carrier iron was added. The cooled digest was neutralized with 40 per cent NaOH, and the precipitated phosphates, sulfates, and hydroxides were thrown down by centrifugation. The iron was extracted by dissolving the precipitate in 7 N HCl and shaking with successive portions of ethyl ether until there was no color left in the aqueous layer. The iron was recovered from the ether by shaking with 1 N HCl, and was electroplated from an oxalate mixture, as previously described,[8] onto a copper disk, which was placed under the mica window of a Kip-type Geiger counter for assay of the radioactivity. In control experiments in which radioiron was added to feces in vitro the recovery was 90 to 100 per cent.[11] In the present study the accuracy of the radioiron measurements was unquestionably lower because of the small amounts of the isotope in the specimens, especially in the later collection periods.

For the estimation of the total amount of radioiron in the circulating hemoglobin, the blood volume of three of the normal men was determined by the Evans blue dye technique.[12] In all the other experiments a blood volume of 80 ml. per kilogram was assumed.

The dogs in which a hemolytic anemia was induced by phenylhydrazine had been members of the colony for several months and were in good health at the beginning of the experiment. They were housed in individual cages with stainless steel bottoms. During the period of observation they were maintained on a diet of bread and milk with a vitamin supplement. The radioactive iron in the feces was determined as described for human subjects.

Sweating was induced by two methods: (1) the subject sat in a cabinet heated by electric light bulbs, with his body wrapped in rubber sheeting in such a way that the sweat drained into a large pan; (2) the subject remained in a steam room in which a temperature of 98° to 104° F. (wet bulb) was attained by running a steam sterilizer with the door open. The sweat was collected either by draping the body in rubber sheeting or by wearing a plastic suit which fitted tightly at the wrists and ankles. The collected sweat was analyzed for radioactive iron by the method used for feces, except that the entire volume of collected sweat was digested and the radioiron was plated directly from the neutralized digest without extraction by ether.[11] Since the sweat contained minimal amounts of iron, 2 mg. of inert carrier iron was added to each sample, before electroplating.

RESULTS

I. *The Excretion of Radioactive Iron in the Feces by Human Subjects.*—A. *Normal individuals:* Three men in good health, with normal red corpuscular constants, were given intravenously, in a single dose, a radioactive iron ascorbate solution containing 5.6 mg. of Fe^{59} and having an activity of 7,200,000 CPM (approximately 18 μc).

The amounts of the isotope which appeared in the peripheral blood and the amounts excreted in the feces over a period of 140 days are shown in Fig. 1. From 70 to 90 per cent of the injected dose promptly appeared in the red cells as hemoglobin. In the experiment on C. M. the collection of feces was begun on the day the iron was injected. The average daily excretion of this initial period and that of the period from the twentieth to the twenty-fourth days are approximately twice as high as the values later in the experiment. In the subsequent periods the daily excretion of the isotope, for all three subjects, averaged 0.01 per cent per day of the amount injected. No significant increase in the excretion at or near the one hundred twentieth day could be detected.

The total iron lost from the body by fecal excretion has been estimated from the radioiron excreted in two different ways. First, if it be assumed that the injected radioiron was evenly mixed with the iron of the hemoglobin, the ratio of *total hemoglobin iron to total hemoglobin radioiron* may be used to estimate the total daily excretion of iron derived from hemoglobin. The calculated values for daily iron excretion, by this formula, are as follows: C. M.—0.46 mg.; J. I.—0.33 mg.; and M. G.—0.38 mg. (Fig. 4, striated columns). The second method of calculation, which assumes complete mixing of the tracer iron with an assumed total body iron of 5 Gm., yields the following values for total daily iron excretion: C. M.—0.52 mg.; J. I.—0.38 mg.; and M. G.—0.48 mg. (Fig. 4, stippled columns). In making these calculations, the values for the initial collection periods were not included in the averages. Because of the assumptions made, the second method gives maximum values for total daily iron excretion.

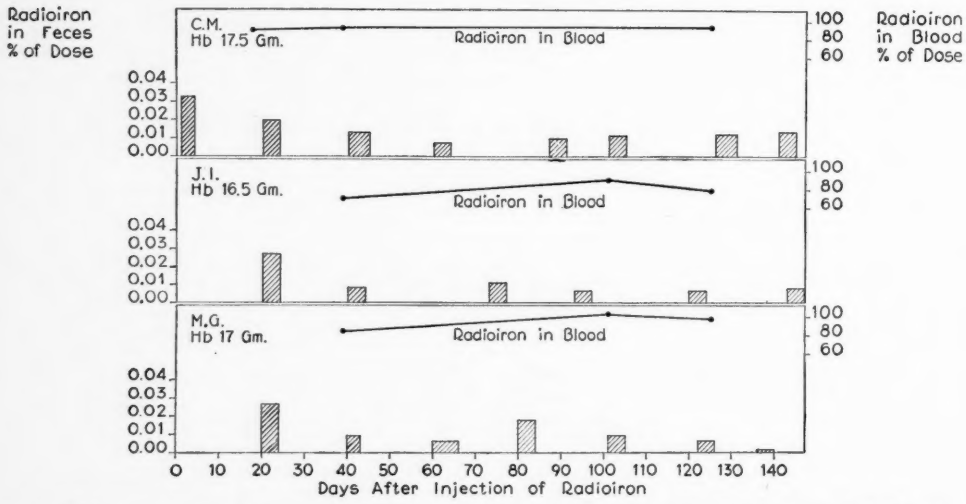


Fig. 1.—The excretion of radioiron by three normal men. Each man received 5.6 mg. Fe^{59} intravenously as ferrous ascorbate. Radioactivity, 7,200,000 CPM. The per cent of the injected radioiron which appeared in the blood is shown by the continuous line at the upper part of each chart. The striated columns show the average radioiron excretion per day for each period, expressed as per cent of the dose.

The fourth normal subject, R. D., was a woman, 48 years of age, whose red cell count and hemoglobin level were within normal limits. R. D. was given intravenously 14 mg. of Fe^{59} + Fe^{55}, with an activity of 2,400,000 CPM.

The level of the isotope in the peripheral blood and the excretion in the feces over a period of 141 days are shown in Fig. 2 (lower chart). As may be seen from the chart, the amount of radioiron excreted during the first two weeks was considerably higher than that excreted in subsequent periods; the amount excreted between the ninety-second and the one hundred forty-first days was lower than at other times. The total fecal iron loss has been calculated in two ways, as was done for the male subjects. From the ratio of *total hemoglobin iron to total hemoglobin radioiron,* the daily excretion of total iron (between the thirty-second and one hundred forty-first days) was estimated to be 0.38 mg. (Fig. 4, striated column). When the iron excretion was calculated from the ratio of the *total body iron to the injected radioiron* (assuming a total body iron of 3 Gm.), the daily iron excretion was estimated to be 0.45 mg. (Fig. 4, stippled column).

A fifth subject, J. N., a man 27 years of age and in good health, with a hemoglobin of 14 Gm., had been given 75 mg. of radioactive iron by mouth for an iron absorption study. Within 12 days, 10 per cent (7.5 mg.) of the isotope had been incorporated in his hemoglobin; the total radioactivity of his circulating blood was 4,300,000 CPM. Several weeks later iron excretion studies were begun. Quantitative collections of feces were made during five periods, beginning with the thirtieth day.

The average daily excretion of radioiron, expressed as per cent of the radioiron in the blood, was 0.016 per cent. The total daily iron excretion, calculated from the ratio of *total hemoglobin iron to total hemoglobin radioiron,* was 0.45 mg. between the sixty-sixth and one hundred fifty-sixth days (Fig. 4). Since J. N. had taken the tracer iron orally and the amount which he had retained (other than for hemoglobin) was not known, an estimate of the iron excretion based on the total body radioiron has not been made.

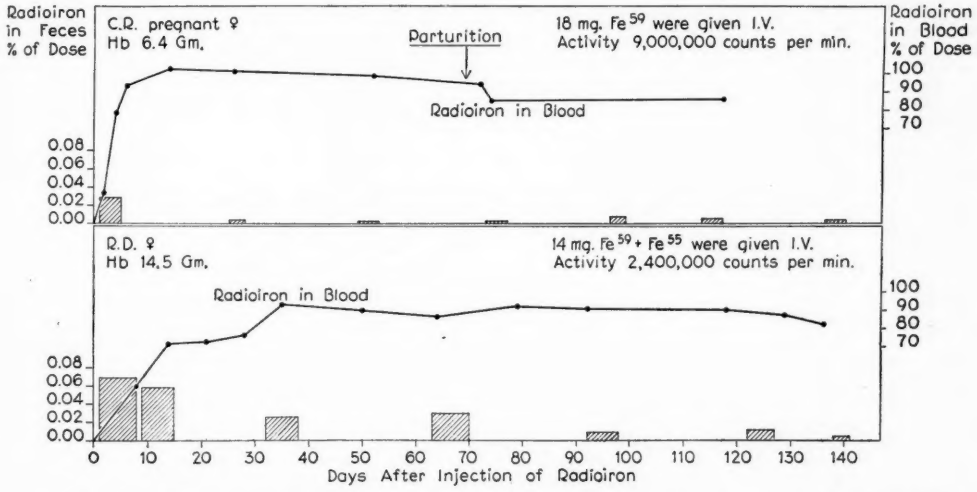


Fig. 2.—The excretion of radioiron by a woman with hypochromic anemia (upper chart) and by a normal woman (lower chart). *Upper chart:* C. R., 28 years of age and pregnant, had a hemoglobin of 6.4 Gm. and a mean corpuscular hemoglobin concentration of 26 per cent. She received 18 mg. Fe^{59} I.V. as ferrous ascorbate and 100 per cent promptly appeared in her circulating blood (continuous line). Each column (along base line) shows the average radioiron excretion per day for the period, expressed as per cent of the dose. *Lower chart:* R. D., 48 years of age and in good health, received 14 mg. Fe^{59} + Fe^{55} intravenously and 80 to 90 per cent of the isotope appeared in her circulating blood (continuous line). The striated columns show the average excretion of radioiron per day for each period, expressed as per cent of the dose.

B. *Patients with hypochromic anemia:* Three women with the anemia of iron deficiency received radioactive iron (two by vein and one by mouth) and made periodic collections of feces, but only one of the three made a quantitative collection between the one hundred fifteenth and the one hundred fortieth days. The results are shown in Fig. 2, upper chart.

C. R., 28 years of age and pregnant, received an intravenous injection of 18 mg. of Fe^{59}, with an activity of 9,000,000 CPM. This patient, who had a hemoglobin of 6 Gm. and a mean corpuscular hemoglobin concentration of 26 per cent, utilized 100 per cent of the injected isotope for hemoglobin. On the sixth day, and thereafter, therapeutic amounts of ferrous gluconate were given orally. After delivery on the sixty-ninth day, the level of the radioiron in the blood fell from 100 to 94 per cent. As may be seen from Fig. 2, the amounts of radioiron excreted by this patient were somewhat less than the amounts

shown for the woman with normal iron stores. There was no increase in the excretion of the isotope at or near the one hundred twentieth day. The total daily fecal iron excretion, calculated from the ratio of the *total hemoglobin iron to total hemoglobin radioiron,* was estimated to be only 0.061 mg. (Fig. 4).

The second woman, M. K., with a hemoglobin of 8.5 Gm. and a mean corpuscular hemoglobin concentration of 29 per cent, was given intravenously 94 mg. of Fe^{59} mixed with Fe^{55} in five daily injections. The total radioactivity was 3,000,000 CPM. At the time administration of the tracer iron was begun the patient was one week post partum. The feces were collected quantitatively for three periods. The amount of radioactive iron excreted per day in the last period (one hundred twenty-sixth and one hundred twenty-seventh days) was not significantly greater than that of an earlier period (thirty-fourth to thirty-ninth days). The total daily iron excretion of this woman, calculated from the ratio of the *total hemoglobin iron to total hemoglobin radioiron,* was 0.032 mg. (Fig. 4).

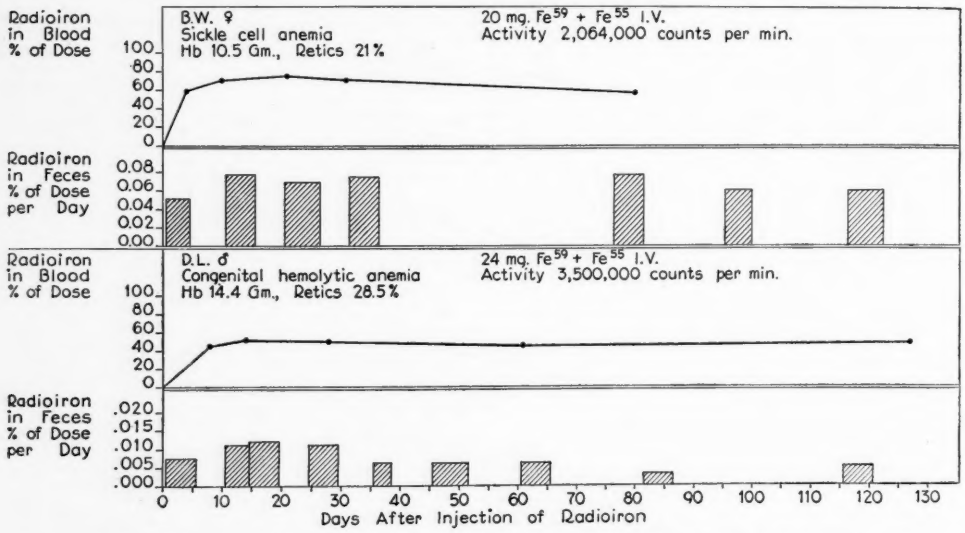


Fig. 3.—The excretion of radioiron by two patients with hemolytic anemia. *Upper chart:* B. W., a woman 26 years of age, with sickle cell anemia, had a hemoglobin of 10.5 Gm. and a reticulocyte count of 21 per cent. She received intravenously 20 mg. Fe^{59} + Fe^{55}. The amount of the isotope in her circulating blood (continuous line) varied from 56 to 74 per cent of the dose. The striated columns show the average amounts of radioiron in the feces per day, expressed as per cent of the dose. *Lower chart:* D. L., a man 18 years of age, with hereditary spherocytosis, had a hemoglobin of 14.4 Gm. and a reticulocyte count of 28.5 per cent. His spleen had not been removed. He received I.V. 24 mg. of Fe^{59} + Fe^{55} in two injections. The amount of the isotope in his circulating blood (continuous line) varied from 44 to 50 per cent. The striated columns show the average amounts of radioiron in the feces per day, expressed as per cent of the dose.

A third woman, M. M., with a hemoglobin of 8 Gm. and a mean corpuscular hemoglobin concentration of 28 per cent, was given by mouth a total of 620 mg. of Fe^{59} mixed with Fe^{55}, in five daily doses. An amount of the isotope equivalent to 99 mg. Fe and having an activity of 3,500,000 CPM had appeared in the blood by the ninth day. Quantitative collections of feces were made during five periods, beginning with the twenty-second day. The amounts of radioiron found in the feces per day during different periods varied from 0.001 to 0.007 per cent of the radioiron in the circulating blood. Because of blood loss from the uterus, the level of radioiron in the blood fell during the period of observation. The total daily iron excretion for M. M., calculated from the ratio of *total hemoglobin iron to total hemoglobin radioiron,* was 0.034 (Fig. 4).

C. *Patients with hemolytic anemia:* The possibility that patients with hemolytic anemia might show an increased excretion of iron was investigated by

giving radioiron intravenously to a woman with sickle cell anemia and to a man with hereditary spherocytosis (spleen had not been removed).

B. W., a woman with sickle cell anemia who had a hemoglobin of 10.5 Gm. and a reticulocyte count of 21 per cent, received an injection of 20 mg. Fe^{59} + Fe^{55} with an activity of 2,064,000 CPM. The collection of feces was made at intervals for 122 days. Results, charted in Fig. 3, show an excretion of iron 5 to 6 times greater than that observed for normal subjects. Moreover, the rate of excretion in the initial period (first to fourth days) was no higher than in later periods. Red cell survival experiments have shown that the average life span of the red cells in this young woman is about 30 days[13]; the experiment, therefore, extended through approximately four erythrocyte life cycles. Excretion, calculated from the ratio of *total hemoglobin iron to total hemoglobin radioiron,* was 1.45

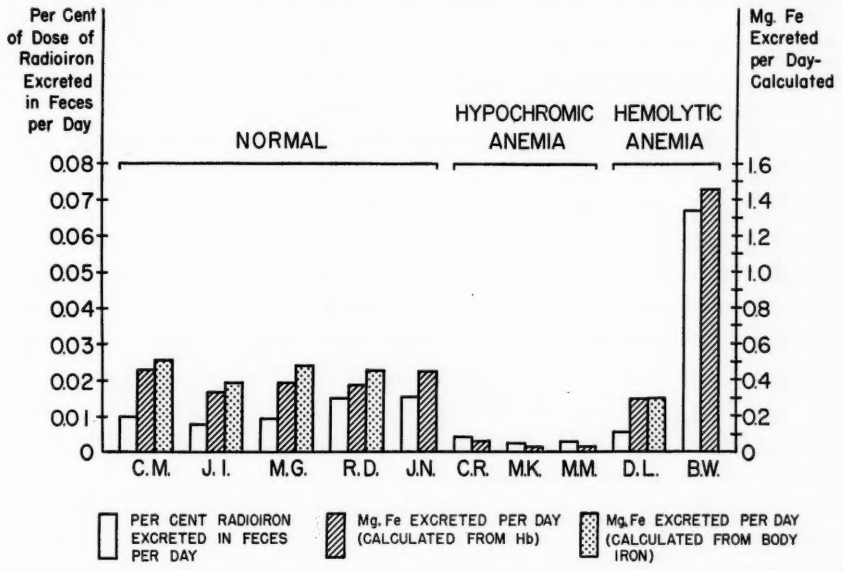


Fig. 4.—The excretion of radioiron (left scale) and the estimated excretion of total iron (right scale) of ten human subjects. The white columns show the radioiron excreted in the feces per day, expressed as per cent of the dose. The striated and stippled columns represent the daily total fecal iron excretion, calculated in two different ways.

The striated columns give a value for total iron excretion based on the ratio of *total hemoglobin iron to total hemoglobin radioiron.*

Example (Calculation for C. M.):

$$\text{Amount of Fe in total Hb} = \frac{17.5}{100} \times 6{,}850 \times 0.00334 = \underset{4{,}000 \text{ mg.}}{4.0 \text{ Gm.}}$$

$$\text{Amount of } Fe^{59} \text{ in total Hb} = \frac{92.2}{100} \times 5.6 = 5.16 \text{ mg.}$$

$$\text{Ratio } \frac{\text{Total Hb Fe}}{\text{Total Hb } Fe^{59}} = \frac{4{,}000}{5.16} = 775$$

$$\text{Amount of } Fe^{59} \text{ excreted daily (av. between thirty-ninth and one hundred forty-fifth days)} = \frac{0.0105}{100} \times 5.6 = 0.00059 \text{ mg.}$$

$$\text{Total Fe excreted in feces daily} = 0.00059 \times 775 = 0.46 \text{ mg.}$$

The stippled columns give a value for total iron excretion based on the ratio of *total body iron to total body radioiron.*

Example (Calculation for C. M.):

$$\text{Amount of Fe in body (assumed)} = \underset{5{,}000 \text{ mg.}}{5 \text{ Gm.}}$$

$$\text{Amount of } Fe^{59} \text{ injected} = 5.6 \text{ mg.}$$

$$\text{Ratio } \frac{\text{Total Body Fe}}{\text{Total Body } Fe^{59}} = \frac{5{,}000}{5.6} = 893$$

$$\text{Amount of } Fe^{59} \text{ excreted daily} = \frac{0.0105}{100} \times 5.6 = 0.00059 \text{ mg.}$$

$$\text{Total Fe excreted in feces daily} = 0.00059 \times 893 = 0.52 \text{ mg.}$$

mg. per day, compared to 0.40 mg. for the normal woman subject (Fig. 4). A calculation based on estimated total body iron has not been made because we had both transfused the patient and removed blood from her on so many occasions that the estimation seemed unwise.

D. L., a man with hereditary spherocytosis who had a hemoglobin of 14.4 Gm. and a reticulocyte count of 28.5 per cent, received 24 mg. of Fe^{59} + Fe^{55}, with an activity of 3,500,000 CPM, intravenously, in two injections. The amounts of radioiron excreted in nine collection periods between the first and one hundred twentieth days approximated normal values (Fig. 3). No significant change in the average daily excretion of the isotope was observed during 120 days. The total daily iron excretion of this subject, calculated from the hemoglobin iron, was only 0.3 mg. or slightly less than that of the normal subjects. The same value was obtained by the ratio of the total body iron (assumed to be 5 Gm.) to the injected radioactive iron (Fig. 4).

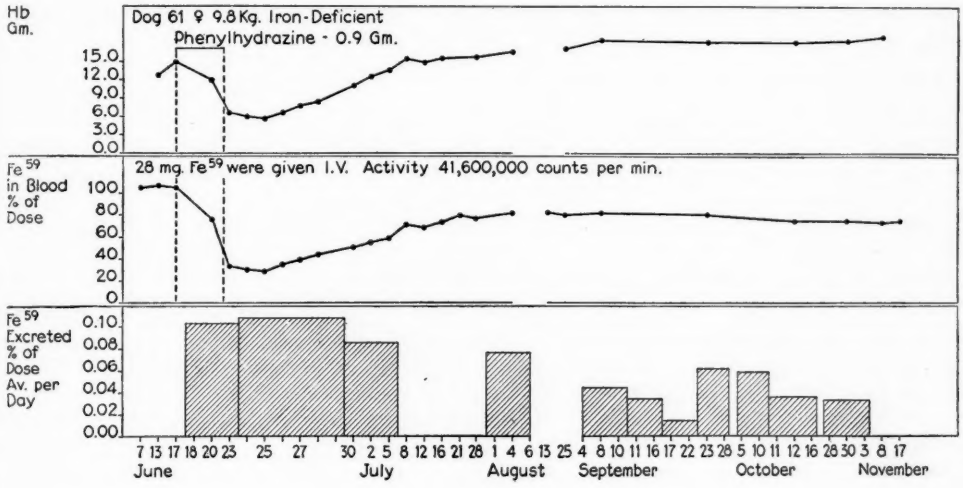
A comparison of the amounts of tracer iron excreted by the ten human subjects is graphically recorded in Fig. 4. The white columns show the average daily excretion of radioactive iron (expressed as per cent of the injected dose) over a time interval when the rate of excretion was relatively constant. The striated columns represent an estimate of the total iron excretion, in milligrams per day, calculated from the hemoglobin iron, and the stippled columns indicate the iron excretion calculated from the total body iron. It is evident that the values for the five normal subjects were similar, that less iron was excreted by the three iron-deficient subjects, and that the value for fecal excretion was high in one of the two patients with hemolytic anemia.

II. *The Excretion of Radioactive Iron by Dogs During a Hemolytic Crisis Induced by Phenylhydrazine.*—The effect of a hemolytic crisis on the capacity of the body to excrete iron was studied by inducing a hemolytic anemia in two dogs with phenylhydrazine. In order to get a maximum concentration of the isotope in the hemoglobin so that observations could be continued for several months, the animals were prepared by making them iron deficient by massive bleedings over a period of eight weeks. Throughout the experiment they were maintained on a bread-and-milk diet with a vitamin supplement.

Dog 61 was a female mongrel, weighing 9.8 kilograms. When the hemoglobin had been reduced to 4 Gm. per 100 ml., 28 mg. of radioiron with an activity of 41,600,000 CPM was injected intravenously in five daily doses. One hundred per cent of the isotope promptly appeared in the peripheral blood (Fig. 5). Beginning on the sixteenth day after the injection, phenylhydrazine was given orally for five days (0.2 Gm. per day for the first three days, then 0.1 Gm. per day for two days). The hemoglobin fell precipitously from 15 Gm. to 5.5 Gm. and the concentration of radioiron in the blood dropped from 100 per cent to 28 per cent of the dose. Quantitative collections of feces were made for 24 days after phenylhydrazine was given. The amounts of the isotope in the feces (Fig. 5) have been recorded as the daily average of six-day periods. Thirty days after the recovery from the acute phase of phenylhydrazine poisoning the feces were collected for six days and the level of the isotope was found to be 0.76 per cent of the dose per day, or almost as high as at the end of the phenylhydrazine period. Between the ninety-fourth and one hundred fifty-third days the radioiron excretion in the feces was followed in order to see whether there might be an increased excretion of the isotope at the end of the life span of the red cells formed during recovery from phenylhydrazine poisoning. As may be seen from Fig. 5, the excretion of radioiron did not rise at or near the one hundred twentieth day after phenylhydrazine. The total iron excreted, calculated from the ratio of the *total hemoglobin iron to total hemoglobin radioiron,* was 0.28 mg. per day after recovery

(ninety-fourth to one hundred fifty-third days), while during the phenylhydrazine period it was 0.38 mg. per day. The total loss of iron in 18 days following phenylhydrazine poisoning was 6.8 mg., or 2.8 per cent of the 240 mg. of iron released during these 18 days by hemoglobin destruction.

Dog 62 was a male spaniel weighing 9.8 kilograms. After its hemoglobin had been reduced to 4.4 Gm. as a result of repeated bleedings, it was given intravenously 28 mg. of radioiron in four daily doses (total activity, 41,600,000 CPM). Ninety per cent of the injected iron appeared in the blood as hemoglobin. When the control period of feces collection was begun, it was found that the feces contained occult blood and hookworm ova. After treatment with tetrachlorethylene, the dog was kept under observation until the feces were parasite-free and guaiac-negative. The fecal excretion of radioiron in a control period was then found to average 0.01 per cent of the injected dose per day.



	During Hemolytic Anemia	After Recovery
Total circulating Hb	$14.9 \times \frac{784}{100} = 116.5$ Gm.	$18.2 \times \frac{872}{100} = 159$ Gm.
Amount of Fe in total Hb	$116.5 \times \frac{0.334}{100} = 0.39$ Gm.	$159 \times \frac{0.334}{100} = 0.53$ Gm.
Amount of Fe^{59} in total Hb	$\frac{100}{100}$ of $28 = 28$ mg.	$\frac{74}{100}$ of $28 = 20.7$ mg.
Ratio: $\frac{\text{Total Hb Fe}}{\text{Total Hb Fe}^{59}}$	$\frac{390}{28} = 13.9$	$\frac{530}{20.7} = 25.6$
Amount of Fe^{59} excreted daily (June 18 to July 5)	$\frac{0.098}{100} \times 28 = 0.0277$ mg.	$\frac{0.0396}{100} \times 28 = 0.0111$ mg. (Sept. 4 to Nov. 3)
Amount of Fe excreted daily (estimated from Hb)	$0.0277 \times 13.9 = 0.385$ mg.	$0.0111 \times 25.6 = 0.284$ mg.

Fig. 5.—The excretion of radioiron by a dog with hemolytic anemia induced by phenylhydrazine. The radioiron was injected on the first day; phenylhydrazine was given by mouth from the sixteenth to the twentieth days. The upper line shows the hemoglobin level, the middle line shows the per cent of the injected radioiron in the circulating blood, and the striated columns show the per cent of the radioiron in the feces per day.

When phenylhydrazine was given for four days (0.2 Gm. daily for two days, 0.1 Gm. daily for two days), the hemoglobin fell from 13 Gm. to 4.6 Gm. and the per cent of radioiron in the hemoglobin fell from 70 per cent of the dose to 26 per cent. The excretion of radioiron during the period of phenylhydrazine poisoning was 0.125 per cent of the dose per day or twelve times that of the control period. During the subsequent recovery period the excretion fell to 0.04 per cent of the dose. The excretion of total iron, calculated from the ratio of *total hemoglobin iron to total hemoglobin radioiron,* is estimated at 0.04 mg. Fe per day for the control period and 0.58 mg. per day for the phenylhydrazine period. The

total amount of iron liberated by hemoglobin destruction in twelve days was 220 mg.; the total iron excretion during the same period was estimated to be 7.4 mg., or 3.5 per cent of the iron liberated.

III. *The Excretion of Radioactive Iron in Sweat by Human Subjects.*—

A. *Normal individuals:* Two men and one woman in good health were given intravenously a single dose of approximately 10 μc of radioactive iron. Immediately after the injection, and on one or more other occasions between the first and the one hundred twentieth days, heavy sweating was induced and the radioactive iron content of the collected sweat was determined.

Only minute traces of the isotope could be detected in the large volumes of sweat collected and the radioiron content could not be correlated either with the volume of the sweat or with the time after the injection. Calculations of iron loss per day were made on the assumption that the same rate of sweat production could be maintained throughout a 24-hour period in order to indicate what the maximum loss might be.

TABLE I. THE LOSS OF RADIOIRON IN SWEAT BY TWO NORMAL MEN AFTER AN INTRAVENOUS DOSE

DAY	PERIOD (HR.)	VOLUME (ML.)	RADIOIRON IN COLLECTED SWEAT (% OF I.V. DOSE)	RADIOIRON PER 24 HR. (μG)	LOSS OF Fe IN SWEAT PER 24 HR. FOR MAXIMAL SWEATING (CALCULATED FROM TOTAL BODY Fe) (MG. Fe)
			Subject C. M.		
1	2⅓	1,010	0.0007	1.38	0.36
7	2	1,050	0.0020	4.61	1.22
36	2	600	0.0020	4.61	1.22
116	2	480	0.0004	0.92	0.24
321	2½	750	0.0032	5.90	1.56
			Subject S. M.		
1	½	82	0.0006	5.18	1.48
28	½	75	0.0006	5.18	1.48

C. M., Hb. 17.5 Gm., received intravenously 19.2 mg. Fe^{59} + Fe^{55} as ferrous ascorbate. Radioactivity 3,500,000 CPM. Sweating was induced by moist heat in a steam room.

Calculation of total iron lost, at maximal rate of sweating. The same rate of sweating was assumed for a 24-hour period. The body iron was assumed to be 5 Gm. The body radioiron was corrected for the excretion of 1.23 per cent of the dose in the urine during the first three hours.

The ratio of total body Fe to body Fe^{59} for C. M. $= \frac{5,000}{19.2 - 0.24} = 264.$

S. M., Hb. 15.7 Gm., received intravenously 18 mg. Fe^{59} + Fe^{55} as ferrous ascorbate. Radioactivity, 4,500,000 CPM. Sweating was induced by dry heat in a cabinet. 2.51 per cent of the radioiron (0.45 mg.) was excreted in the urine in the first hour.

The ratio of total body Fe to body Fe^{59} for S. M. $= \frac{5,000}{18 - 0.45} = 285.$

C. M. was given 19.2 mg. of Fe^{59} + Fe^{55} intravenously as ferrous ascorbate, with an activity of 3,500,000 CPM; immediately after the injection he made a collection of 1,010 ml. of sweat by spending two hours in a steam room. The radioiron found in the sweat (Table I) was only 0.0007 per cent of the amount injected. The amount of the isotope excreted in the urine during the same period was 1.23 per cent and the level observed in the plasma 14 hours later was 4.06 per cent of the dose. Table I shows the results of four subsequent collections of sweat, made on the seventh, thirty-sixth, one hundred sixteenth, and three hundred twenty-first days. An estimate of the total iron loss in sweat has been attempted by assuming a complete mixing of the isotope with the body iron stores so that the ratio of total body iron to body radioiron is used as a conversion factor. The total

estimated iron loss per 24 hours (at the observed rate of sweating) ranged from 0.24 to 1.56 mg. (Table I). It should be emphasized that these values represent an estimate of iron loss at a maximal rate of sweating. Since sweating was induced by extreme methods which, in several instances, almost caused fainting, it could not have been tolerated at this rate for more than short periods of time.

S. M. was given 18 mg. of Fe^{59} + Fe^{55} intravenously as ferrous ascorbate with a total activity of 4,500,000 CPM; immediately after the injection he entered a cabinet heated by electric light bulbs (temperature 130° F.) and during 33 minutes collected 82 ml. of sweat. The radioiron content was 0.0006 per cent of the dose (Table I). The concentration of the isotope in the urine for the same period was 2.5 per cent or 4,500 times as great. On the twenty-eighth day a second collection of sweat was made (Table I). Although the volumes of sweat collected by S. M. (under dry heat) were only one-twelfth the volumes collected by C. M., the amounts of iron lost during the collection period were similar. If, however, the values are computed as micrograms of Fe^{59} per liter of sweat, much higher values are obtained for S. M. Under dry heat a more concentrated sweat was collected because of partial evaporation of the sweat from the skin surface. The maximal iron loss in sweat, estimated from the ratio of total body iron to body radioiron, was found to be 1.48 mg. per day (Table I).

R. D., the woman subject, received an intravenous injection of 19 mg. of Fe^{59} + Fe^{55}, with an activity of 4,600,000 CPM; she made collections of sweat immediately after the injection and on eight other occasions between the first and one hundred thirty-fourth days (Table II). The initial collection, totaling 400 ml., was made in a steam room and contained 0.0017 per cent of the dose—a larger amount than was found in subsequent collections. The amount of the isotope excreted in the urine during the first hour was 2.12 per cent of the dose. With this subject both moist heat and dry heat were used on different occasions to induce sweating; the concentration of radioiron per liter of sweat did not show any correlation with the type of heat used. The maximal iron loss in sweat per 24 hours has been calculated from the ratio of total body iron to body radioiron, assuming a body iron store of 3 Gm. (Table II). Beginning on the ninety-sixth day, ferrous gluconate in therapeutic amounts (156 mg. Fe per day) was taken by mouth until the one hundred thirty-fourth day in order to see whether some of the radioiron liberated by the physiologic destruction of the red cells might be replaced by newly

TABLE II. THE LOSS OF RADIOIRON IN SWEAT BY A NORMAL WOMAN AFTER AN INTRAVENOUS DOSE

DAY	PERIOD (HR.)	VOLUME (ML.)	RADIOIRON IN COLLECTED SWEAT (% OF I.V. DOSE)	RADIOIRON PER 24 HR. (μG)	LOSS OF IRON IN SWEAT PER 24 HR. FOR MAXIMAL SWEATING (CALCULATED FROM TOTAL BODY Fe) (MG. Fe)
1	1	400	0.0017	7.78	1.26
7	1	310	0.0003	1.34	0.22
15	1	385	0.0007	3.19	0.52
28	1	90	0.0001	0.46	0.07
42	1	616	0.0003	1.37	0.22
96	1	405	0.0007	3.19	0.52
116	1	350	0.0005	2.28	0.38
125	1	390	0.0008	3.65	0.59
134	1	675	0.0004	1.82	0.29

R. D., Hb. 13.5 Gm., received intravenously 19 mg. Fe^{59} + Fe^{55} as ferrous ascorbate. Radioactivity, 4,600,000 CPM.

On Days 1, 125, and 134, sweating was induced by moist heat in a steam room. On all the other days dry heat was used. *Calculation of the total iron lost, at maximal rate of sweating.* The same rate of sweating was assumed for a 24-hour period. The body iron was assumed to be 3 Gm. The body radioiron was corrected for the excretion of 2.12 per cent of the dose in the urine during the first one and one-half hours.

The ratio of total body Fe to body $Fe^{59} = \frac{3{,}000}{19 - 0.4} = 161.$

absorbed inert iron. A moderate depression of the radioactive iron concentration of the blood occurred (from 18 mg. to 16 mg. between the one hundred thirtieth and the one hundred thirty-fourth days), but the excretion of the isotope in the sweat did not increase.

B. *Patient with hypoplastic anemia:* L. H., a man 19 years of age who had been treated for refractory anemia for two years and had received a total of fifty-two whole blood transfusions of 500 ml. each, was given 1.7 mg. Fe^{59} as ferrous ascorbate intravenously; five days later a collection of 550 ml. of sweat was made (Table III). The radioiron content was 0.003 per cent of the dose—somewhat higher than that observed for the three normal subjects.

Twenty-five days later, sweat was again collected, during three separate periods. Not only did the volume of sweat secreted become greater in each successive period but the concentration of the isotope increased (Table III). Our estimate of the total iron loss in sweat in 24 hours by this patient (Table III, Column 6) is actually of little value. Since the patient, before the beginning of the study, had received 52 units of blood, containing approximately 10 Gm. of iron, his total body iron stores had probably been increased to about 15 Gm. The assumption of complete mixing of the isotopic iron with this abnormal store of iron is probably less valid than in the case of normal individuals. Moreover, the variation in the loss of radioiron in successive sweating periods on the same day shows the inaccuracy of attempting to calculate the 24-hour excretion of iron from a given volume of sweat. The excretion of total iron for the same 24-hour period has been estimated at three different values—0.7 mg., 7.2 mg., and 12.2 mg., depending on which period of sweating was used as a basis (Table III, Column 6). The increasing amounts of radioiron lost by L. H. in successive periods of sweating strongly suggest that the iron in sweat may come from desquamated epithelial cells rather than from secretion by the sweat glands.

TABLE III. THE LOSS OF RADIOIRON IN SWEAT BY A PATIENT WITH HYPOPLASTIC ANEMIA

DAY	PERIOD	VOLUME (ML.)	RADIOIRON IN COLLECTED SWEAT (% OF I.V. DOSE)	RADIOIRON PER 24 HR. (μG)	LOSS OF IRON IN SWEAT PER 24 HR. FOR MAXIMAL SWEATING (ESTIMATED FROM TOTAL BODY Fe) (MG. Fe)
5	2 hr.	550	0.0030	0.61	5.39
25					
A.M.					
	1st hr.	200	0.0002	0.08	0.72
	Next 45 min.	320	0.0015	0.82	7.18
P.M.					
	1 hr.	550	0.0034	1.39	12.20

L. H., Hb. 6.4 Gm., received intravenously 1.7 mg. Fe^{59} as ferrous ascorbate. Radioactivity, 3,800,000 CPM. Sweating was induced by moist heat in a steam room. L. H. had received transfusions of 52 units of blood before the beginning of the study. His body iron stores had been increased to an estimated 15 Gm. by these transfusions. *Calculation of total iron lost at maximal rate of sweating.* The same rate of sweating was assumed for a 24-hour period. The total body iron was assumed to be 15 Gm.

The ratio of total body Fe to body Fe^{59} for L. H. $= \frac{15{,}000}{1.7} = 8{,}800.$

IV. *The Appearance of Tracer Iron in Saliva and Hair Clippings.*—

When iron ascorbate is given intravenously, a strong taste of iron is noticed. In order to see whether significant amounts of iron might appear in the saliva after intravenous injection, one subject chewed paraffin and collected 17 ml. of saliva during two hours after the isotope was injected. The amount of radioiron found was the same as that of the much larger volume of sweat (1,010 ml.) collected during the same interval (Table IV).

TABLE IV. RADIOACTIVE IRON CONTENT OF SALIVA AFTER INTRAVENOUS INJECTION

SUBJECT	TIME AFTER RADIOIRON INJECTION	Fe^{59} IN SALIVA 17 ML. (% OF I.V. DOSE)	Fe^{59} IN SWEAT 1,010 ML. (% OF I.V. DOSE)	Fe^{59} IN URINE (% OF I.V. DOSE)	Fe^{59} IN PLASMA (% OF I.V. DOSE)
C. M.	0-2 hr.	0.0006	0.0007	1.23	--
	14 hr.	--	--	--	4.06

C. M. received 19.2 mg. Fe^{59} + Fe^{55} intravenously as ferrous ascorbate. Radioactivity, 3,500,000 CPM (Table I).

As a part of the study of the excretion of radioiron by the skin, an attempt was made to determine the amount of the isotope in hair clippings. In the experiment on one of the normal men, the hair clippings on the seventy-fifth and one hundred eighth days after injection of the isotope were assayed for radioactivity. The results are shown in Table V. On the one hundred eighth day the clippings were thoroughly extracted with hydrochloric acid to remove iron from adsorbed perspiration. The results show that small but detectable amounts of body iron are lost in the hair clippings and that (in one assay) only one-fifth of the amount of tracer iron in the hair remained after extraction with hydrochloric acid (Table V).

TABLE V. RADIOACTIVE IRON CONTENT OF HAIR CLIPPINGS

SUBJECT	TIME AFTER RADIOIRON INJECTION	Fe^{59} IN BLOOD (% OF I.V. DOSE)	WT. OF HAIR CLIPPINGS (GM.)	Fe^{59} IN HAIR CLIPPINGS			
				% OF I.V. DOSE		μG PER GM.	
				TOTAL Fe^{59}	HCl EXTRACTABLE Fe^{59}	TOTAL Fe^{59}	HCl EXTRACTABLE Fe^{59}
C. M.	75 days	89	9.0	0.0018	--	0.38	--
	108 days	89	4.3	0.0010	0.0008	0.04	0.032

C. M. received 19.2 mg. Fe^{59} + Fe^{55} intravenously as ferrous ascorbate. Radioactivity, 3,500,000 CPM (Table I).

DISCUSSION

The radioactive iron technique is ideally suited to the study of iron excretion since it permits differentiation of excreted iron from iron present either because of contamination or because it was not absorbed from food. To our knowledge, the only comparable study of fecal radioiron excretion is that made by Hahn and co-workers,[1] in 1939, on the dog. These investigators noted a short initial period of accelerated excretion after the injection of radioiron, followed by a steady and low output of 0.05 to 0.4 mg. of the isotope per day. The radioiron used was of low specific activity. By giving radioiron of relatively high specific activity to human subjects we have been able to follow the excretion of iron for long periods—120 to 140 days.

The difficulties of determining iron in feces have frequently been pointed out in the literature.[14] The technique for determining radioactive iron in feces which we have used will determine the isotope with an accuracy of ± 10 per cent when sufficient activity is present for counting. In spite of the relatively high activity of the Fe^{59} injected, some of the samples of feces toward the end of the period of observation had less than an optimal activity for counting, so that the error of the method was increased. Since, however, a very high level of radioactivity was maintained in the blood, any increased excretion at the time of the destruction of considerable numbers of red cells containing the isotope

would have been easily detected. Because of the increase in the error of the method in the later collection periods and the physiologic variation in the amounts excreted from day to day, occasional slight increases in the excretion of the isotope observed between the one hundredth and one hundred fortieth days (Figs. 1 to 3) are of questionable significance.

The most impressive finding which emerges from our study is the minuteness of the iron loss by way of the bowel. In the experiment on B. W., the young woman with sickle cell anemia, the rate of excretion of the isotope was five to six times that of the normal subjects. During the 122 days of observation an estimated total of 1.6 per cent of the injected dose was excreted in the feces. Yet even in the case of B. W., assuming that the observed rate of excretion were to continue indefinitely, it would require 21 years for the total dose of 20 mg. to be excreted. That the body conserves its iron even during a severe hemolytic crisis is shown by the experiment on Dog 61. During a period in which the iron released by the destruction of hemoglobin was approximately 240 mg., the calculated total excretion of iron was only 6.8 mg.—or 2.8 per cent of the iron released. It is not surprising, then, that we were unable to demonstrate an increased excretion of the isotope at or near the one hundred twentieth day after its incorporation in the red cells.

The mechanism by which iron is excreted by the intestinal tract remains obscure, but there are three possibilities: (1) Bile contains a small amount of iron, presumably derived from the catabolism of hemoglobin. In the dog it has been estimated that 3 per cent of the iron freed by hemolysis is excreted in bile.[15] The iron which reaches the intestinal tract in this manner is almost certainly not completely absorbed. One would expect, therefore, that a greater percentage of any given dose of radioiron might be found in the feces of at least some patients with hemolytic anemia (as in B. W.). (2) The colon may actually excrete a small quantity of iron. There is no direct evidence to support this possibility, but still it cannot be denied with finality. If excretion were an active process, one would expect fecal radioiron to be found in greatest amounts during the first few days after injection of the isotope—i.e., before the injected iron had been synthesized into hemoglobin or myoglobin, or stored in the tissues. Actually, in the normal subjects, there was a greater amount of the isotope excreted during the first few days than was found later, but this increased quantity may have come from bile. Furthermore, in the patients with hemolytic anemia, the fecal radioiron was as high several weeks after injection as it was initially. (3) A portion of fecal iron must be derived from cells desquamated by or lost into the intestinal tract. A few red corpuscles and granulocytes can always be found during the microscopic examination of any fecal specimen. It has been postulated that lymphocytes are "excreted," largely by the Peyer's patches, into the intestinal lumen. Intestinal epithelial cells may have a very rapid turnover rate with an average life of only 1.37 to 1.5 days.[16] All of these cells contain iron. One can be fairly certain that the first and third possibilities listed provide some of the iron in feces; true excretion by the colon seems much less likely.

The very small amounts of radioiron which appeared in sweat show that, in the normal individual, the skin is not important as an organ of iron excretion. The difficulties of avoiding contamination in studies in which the iron of sweat is chemically determined are well known.[5] Although large volumes of sweat were collected and analyzed in the present study, the amounts of radioactive iron present were, in all cases, lower than the optimal activity for counting. There is, consequently, a larger error in the figures for the radioiron content of sweat than in those for feces. It is also questionable whether we are justified in attempting to calculate the iron concentration per liter of sweat or for a 24-hour period. The iron may come largely from desquamated epithelial cells, and the amount lost through the skin may be quite independent of the volume of sweat secreted.

While the data presented demonstrate that only small amounts of iron are lost from dermal surfaces and from the bowel, they also emphasize the fact that some loss does occur. It is difficult to calculate from the values for radioiron excretion how much total iron excretion there may be, yet that value must be known before one can evaluate the nutritional importance of the small iron loss which does occur. In an attempt to estimate total excretion, we multiplied the value for radioiron loss by two different ratios:

(a) $\frac{\text{Total hemoglobin iron}}{\text{Radioiron in circulating hemoglobin}}$

(b) $\frac{\text{Estimated total body iron}}{\text{Total radioiron in the body}}$

The figures derived from this kind of calculation provide at best only a rough estimate of total iron excretion, but it is of interest that (a) for normal subjects, the estimated fecal loss of iron was about 0.5 mg. per day; (b) in the few iron-deficient patients studied, no evidence of a metabolic disorder leading to increased excretion was found. One can be reasonably sure from these calculations that if total iron excretion in urine, sweat, and feces were estimated at 0.5 to 1 mg. per day, the estimate would not be far in error. Small as this quantity is, however, it is of considerable nutritional importance. There is evidence to indicate that the normal person, consuming a diet with about 15 mg. of food iron per day, probably absorbs less than 10 per cent of the ingested iron.[17] There is less of a positive iron balance, therefore, than many workers have supposed, and statements to the effect that positive balance can be maintained on diets which provide only a few milligrams of iron are almost certainly incorrect. By the same token, adults who have absorptive defects or an inadequate diet over a prolonged period of years probably do develop iron deficiency on a nutritional basis, even in the absence of blood loss.

SUMMARY

A systematic study of the excretion of iron in feces and sweat by human subjects over a period of 140 days has been made by injecting radioactive iron parenterally as a tracer.

The results of the study of radioiron excretion in the feces show that: (a) When 5 to 14 mg. of radioiron was injected intravenously into four normal individuals, the average daily excretion over a period of 100 days was 0.01 per cent of the dose, or 0.4 to 2.0 μg per day. (b) In three women with an iron deficiency, the daily excretion averaged 0.002 to 0.004 per cent of the dose. (c) In a woman with a hemolytic anemia, the daily excretion averaged 0.07 per cent of the dose; in a second subject with hemolytic anemia, the excretion of the isotope was at the same level as that of the normal persons. (d) No increase in the excretion of tracer iron at or near the one hundred twentieth day after injection was observed. (e) The initial excretion of the isotope by the normal subjects (first to twentieth day) was greater than in later periods.

The total iron excreted per day in the feces has been calculated from the ratio of the radioiron to the hemoglobin iron and from the ratio of the radioiron to the total body iron. (a) For the normal subjects, the calculated excretion in the feces was 0.33 to 0.52 mg. Fe per day. (b) For the iron-deficient subjects, the total iron excretion was calculated to be 0.03 to 0.06 mg. Fe per day. (c) For a woman with hemolytic anemia, the calculated total iron excretion was 1.45 mg. per day; for a man with hemolytic anemia, 0.30 mg. per day.

In two dogs in which the excretion of radioactive iron in feces was studied during a hemolytic anemia induced by phenylhydrazine, the amounts of tracer iron excreted were 2.8 and 3.5 per cent of the iron released by hemoglobin destruction.

In large amounts (75 to 1,050 ml.) of sweat, collected at intervals for 140 days by three healthy human subjects who had received radioactive iron parenterally, only traces of the istotope were found. No increase in the radioiron excretion in the sweat at or near the one hundred twentieth day was observed.

A man with hypoplastic anemia who had received 52 whole blood transfusions excreted amounts of radioactive iron in his sweat comparable to the excretion observed in normal subjects.

While the mammalian body does excrete iron with difficulty, the amounts lost are of physiologic and nutritional importance.

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THE USE OF Cr^{51} AND Fe^{59} IN A COMBINED PROCEDURE TO STUDY ERYTHROCYTE PRODUCTION AND DESTRUCTION IN NORMAL HUMAN SUBJECTS AND IN PATIENTS WITH HEMOLYTIC OR APLASTIC ANEMIA

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THE clearance of iron from plasma, the turnover rate of plasma iron, and the rate of utilization of iron by newly formed erythrocytes are established indices of erythrocyte production, as measured by the radioiron dilution technique,[1-3] in man and many experimental animals. Determination of the rate of erythrocyte destruction, based on these procedures, can also be made, but is ordinarily much less accurate. This is particularly true when the average lifespan of the red cell is estimated from the plasma iron turnover rate.[4] The lifespan of newly formed erythrocytes can be measured accurately with radioiron if the recycling of the radioiron can be blocked by "iron-loading."[5] This technique has been more successful in laboratory animals than in human subjects.

In our experience, more accurate information concerning the state of erythrocyte production and destruction in human subjects may be obtained by combining the radioiron with the radiochromium-erythrocyte labeling method, which has been shown to be a simple, reliable, and adaptable technique for measuring the blood volume[6] and the survival time of a mixed population of erythrocytes in healthy human subjects as well as in patients with various hematologic diseases.[7-10] In addition to giving a more quantitative index of red cell destruction, the Cr^{51}-labeling method affords a simple and precise determination of the blood volume that is necessary for calculating the plasma iron turnover rate and per cent of iron utilized in the formation of newly formed erythrocytes.

The combined procedure is relatively simple, provided an efficient well-type scintillation counter, equipped with an adequate pulse height selector, is available. Since there is a marked difference in intensity of the gamma emissions from the two isotopes, it is possible to differentiate the radioactivity of Fe^{59} from that of Cr^{51} in the same blood sample.

The purpose of this paper is to (1) describe the combined labeling procedure; (2) illustrate its application with results from a normal subject, one subject with a hemolytic anemia, and one with an aplastic anemia; and (3) discuss the findings, emphasizing the importance of the information that has been obtained, as well as the limitations of some of the values.

METHODS AND MATERIALS

A. *Iron Plasma Clearance and Plasma Iron Turnover.*—The radioiron used in these studies was Fe^{59}, obtained from Oak Ridge as $FeCl_3$ in 0.080 N HCl solution, free of Fe^{55}.

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It was adjusted to pH 6.0 and autoclaved before use. The specific activity was 4.57 mc. per milligram. The method of Huff[2] was used to determine the plasma clearance and plasma iron turnover. Two to four microcuries of Fe^{59} was added to approximately 20 ml. of plasma, and the mixture incubated at 37° C. for 30 minutes. The radioactivity (cpm) in an aliquot was counted and then the plasma-Fe^{59} mixture was injected into a large arm vein by means of a heparinized syringe and No. 18 needle. Heparinized blood samples were withdrawn from the subject at appropriate intervals depending on the expected rate of plasma iron clearance. The hematocrit of each sample was recorded after centrifuging for 30 minutes at 2,500 r.p.m. in an International Centrifuge, Size 1. One milliliter plasma aliquots were pipetted in duplicate and counted in a well-type scintillation counter, Nancy Wood Model 1050A. The clearance rate of radioiron from the plasma was calculated by plotting semi-logarithmically the total counts per minute in the plasma of 3 ml. of whole blood against the time in hours. Preliminary observations revealed that there was no significant difference in the half-time clearance if the data were corrected for the small number of counts present in the red cells during the length of time necessary to determine the plasma clearance.

Plasma iron and serum iron-binding capacity were measured by the methods of Barkan and Walker[11] and Rath and Finch,[12] respectively. Normal values for these determinations in our laboratory are 70γ per cent to 125γ per cent for plasma iron and 175γ per cent to 225γ per cent for serum iron-binding capacity.

Plasma iron turnover in milligrams per kilogram per day was calculated by the formula[2]:

$$\frac{0.693 \times \text{plasma iron mg./ml.} \times \text{plasma volume} \times 24}{T\tfrac{1}{2} \text{ in hours} \times \text{wt. in kg.}}$$

The plasma volume was measured by the Cr^{51} technique (vide infra).[6]

B. *Per cent Iron Utilization.*—The amount of radioiron that appeared in newly formed red cells was determined in the following manner. Heparinized samples of whole blood were withdrawn from the subjects at appropriate intervals, usually every other day, starting the morning after the plasma clearance had been determined. The hematocrit of each sample was recorded. Three milliliter aliquots of each whole blood sample were pipetted in duplicate and stored in the cold. After several days of collection, all the samples were counted at the same time, thus obviating the use of a radioiron standard. The counts per minute per millimeter of red cells were calculated from the hematocrit. Inasmuch as no significant radioactivity was present in the plasma after 24 hours, we did not measure the radioactivity in washed red cells.

Per cent iron utilization was calculated by the formula:[1]

$$\text{Per cent Fe utilization} = \frac{\text{counts/min./ml. red cells} \times \text{red cell volume} \times 100}{\text{total counts/min. injected}}$$

The red cell volume was determined by the Cr^{51} technique (vide infra).[6]

C. *Cr^{51} Blood Volume and Survival Time.*—Radiochromium, $Na_2Cr^{51}O_4$, was obtained from Oak Ridge through Abbott Laboratories, Chicago, Illinois. The material had a specific activity of 1.66 mc. per milligram. Blood volume was determined by the method of Sterling and Gray,[6] using the formula:

$$\text{Total blood volume in ml.} = \frac{\text{counts/min./ml. preinjection sample} \times \text{ml. blood injected}}{\text{counts/min./ml. postinjection sample}}$$

The postinjection sample was drawn in 1 hour. Red cell volume was calculated by multiplying the total blood volume by the uncorrected venous blood hematocrit. Plasma volume was estimated by subtracting the red cell volume from the total blood volume.

In our laboratory normal values for these determinations are:

Total blood volume = 50 to 80 ml./kg.
Red cell volume = 23 to 36 ml./kg.
Plasma volume = 27 to 44 ml./kg.

Cr^{51}-labeled erythrocyte survival studies were performed as described previously.[7, 8] The apparent half survival time was found to be 33 ± 3.2 days for normal human subjects. Apparent survival time implies that no correction has been made for Cr^{51} elution.

Under ordinary circumstances, it is our practice to delay the Cr^{51}-labeling procedure for several days after the administration of the Fe^{59} in order to keep the counting and calculation data as simple as possible. After Cr^{51} has been given, the determination of radioactivity from each of the isotopes may be calculated from the same whole blood sample by means of the following formulas:

$$C_\alpha = \frac{T_\alpha - K_F\, T_\beta}{1 - \frac{K_F}{K_C}} \quad \text{and} \quad F_\beta = T_\beta - \frac{T_\alpha - K_F\, T_\beta}{K_C - K_F} \quad \text{or} \quad F_\beta = T_\beta - \frac{C_\alpha}{K_C}$$

These formulas are derived in the following manner:

Let C_α = Portion of count at pulse height α due to Cr^{51},
Let F_α = Portion of count at pulse height α due to Fe^{59},
Let C_β = Portion of count at pulse height β due to Cr^{51},
Let F_β = Portion of count at pulse height β due to Fe^{59},
Let T_α = Total count at pulse height α,
Let T_β = Total count at pulse height β.

$\therefore$ 1. $T_\alpha = C_\alpha + F_\alpha$
2. $T_\beta = C_\beta + F_\beta$

and by definition

3. $K_C = \frac{C_\alpha}{C_\beta}$
4. $K_F = \frac{F_\alpha}{F_\beta}$

These equations have four unknown quantities: C_α, C_β, F_α, and F_β. The constants, K_C and K_F, are determined by using counting tubes containing only Cr^{51} and Fe^{59}. T_α and T_β are the actual counts obtained in the mixed samples at the respective pulse heights. The formulas for C_α and F_β are derived by solving the four equations, and may be applied by experimentally determining the constants K_C and K_F.

D. *Counting Equipment and Data.*—The radioactivity of Fe^{59} and Cr^{51} in these experiments was counted in a well-type scintillation counter with a Tl-activated NaI crystal. The scaler* was equipped with a Schmitt-type discriminator that can be preset by the operator to select electrical impulses only above a desired energy level, and to eliminate background. We found this discriminator useful enough to differentiate between the emissions from radioiron and radiochromium. Undoubtedly, a single channel pulse analyzer would be necessary for differentiating between isotopes with energies of closer intensity.

The radioactivity of Fe^{59} and Cr^{51} used in these studies is substantially below the maximum permissible amounts in human subjects.†

Following the Cr^{51}-erythrocyte tagging procedure, a postinjection sample of heparinized blood was taken at 1 hour to determine the blood volume. Then samples were withdrawn at appropriate intervals, usually every other day, for determination of the Cr^{51} survival time and completion of the Fe^{59} utilization curve. Ordinarily, all the samples were refrigerated and counted at the same time. With a Cr^{51} standard, a pulse height (α) was selected at which Cr^{51} could be counted at maximum efficiency. A second pulse height (β) was selected that, for all practical purposes, eliminated impulses from Cr^{51} but still counted Fe^{59} at a reasonable efficiency. It is suggested that a Cr^{51} standard, with approximately 50,000 cpm at the lower pulse height, be used in the determination of the second pulse height. It should be noted that even with the use of our relatively crude type of pulse height selector, the $1 - \frac{K_F}{K_C}$ denominator of the C_α formula was almost always very close to 1.0, thus simplifying the formula to $C_\alpha = T_\alpha - K_F\, T\beta$.

*Atomic Instrument Co., Model No. 1050A, Cambridge, Mass.
†Handbook 52, United States Department of Commerce, National Bureau of Standards.

RESULTS

A. *Normal Subjects.*—Typical curves for Fe^{59} plasma clearance, per cent Fe^{59} utilization, and Cr^{51} erythrocyte survival for a normal human subject are shown in Fig. 1. The half-time for Fe^{59}-plasma clearance in this case was approximately 1 hour, slightly shorter than the average half-time reported by Huff and co-workers[2] and Wasserman and associates.[3] In a series of 6 normal human subjects, the half-time Fe^{59}-plasma clearance ranged from 1.0 to 2.0 hours. The per cent Fe utilization curve (Fig. 1) is similar to those reported by Finch and associates.[1] However, in the same series of 6 subjects, the maximum Fe utilization occurred within 7 days in 2 subjects and within 12 days in all of them. The maximum uptake ranged between 80 and 95 per cent of the injected radioiron. The minor differences in the iron plasma clearance and per cent iron utilization reported here may be due, in part, to the higher specific activity of the Fe^{59} used in these experiments. We have noted no significant differences in the Cr^{51} survival curves determined by this combined method as compared with data from our earlier studies.

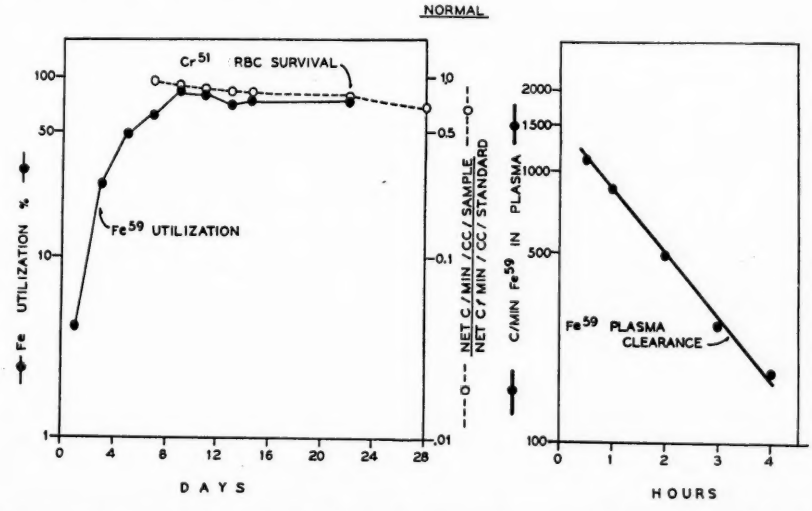

Fig. 1.—Typical curves for radioiron plasma clearance, radioiron utilization by newly formed erythrocytes, and survival time of a mixed population of radiochromium-tagged erythrocytes in a normal subject.

In our normal subjects, Fe^{59}-plasma turnover ranged from 0.38 to 0.65 mg. per kilogram per day. Again, these values are somewhat higher than those reported by Huff and colleagues.[2] This was to be expected in view of the more rapid Fe^{59}-plasma clearance values.

B. *Hemolytic Anemia.*—Data from a subject with an acquired hemolytic anemia are presented in Fig. 2. The very rapid Fe^{59}-plasma clearance is obvious and needs no amplification except to point out that, in our experience, plasma clearance of Fe^{59} may be so rapid in cases of hemolytic anemia that no reliable quantitative data can be based on this measurement alone or on the Fe-plasma turnover data. The per cent Fe^{59} utilization, on the other hand, is, on the whole,

reliable and reproducible. The curve demonstrates a more rapid than normal uptake of the radioiron which, however, never reaches the maximum levels achieved in normal human beings.

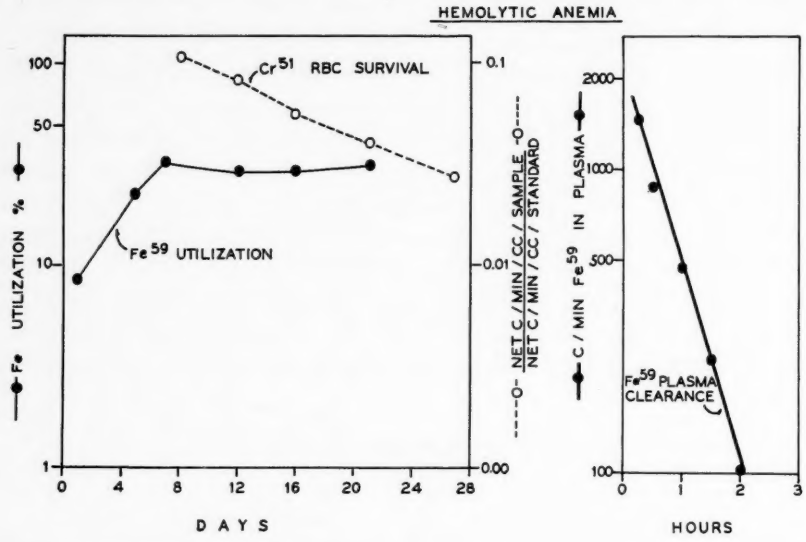

Fig. 2.—Typical curves for radioiron plasma clearance, radioiron utilization by newly formed erythrocytes, and survival time of a mixed population of radiochromium-tagged erythrocytes in a subject with a hemolytic anemia.

C. *Aplastic Anemia.*—Results from a patient with idiopathic aplastic anemia are given in Fig. 3. The delayed Fe^{59}-plasma clearance, poor utilization of the radioiron, and relatively normal survival curve are in accord with the usual findings in aplastic anemia. The subject selected for this illustration did not have a complete saturation of plasma iron-carrying protein (serum Fe 150γ per cent, iron-binding capacity 100γ per cent), and, therefore, the delayed Fe-plasma clearance time is probably an accurate index of the rate of clearance of iron. However, in many instances, patients with aplastic anemia have no demonstrable iron-binding capacity. In these instances, iron plasma clearance times, as measured by the method used in these studies, have no accurate quantitative significance. The same statement holds for those subjects with hemolytic anemias whose plasma iron-carrying protein is saturated. For the same reason, the Fe-plasma turnover rate is unreliable. However, the per cent Fe^{59} utilized in aplastic anemia is ordinarily a very accurate and useful index of erythrocyte formation.

We have found the dual analysis of erythrocyte formation, as measured by per cent Fe^{59} utilization, and erythrocyte destruction, as measured by Cr^{51} survival time, to be exceedingly important in establishing a possible course of treatment for patients with this blood dyscrasia. The demonstration of accelerated blood destruction by the Cr^{51} technique is a strong indication for a therapeutic trial of cortisone or ACTH, and perhaps splenectomy. It should be emphasized,

however, that one must be certain that the accelerated Cr^{51} curve really represents blood destruction and not active gastrointestinal bleeding, an all too frequent event in subjects with aplastic anemia who have markedly reduced platelet levels.

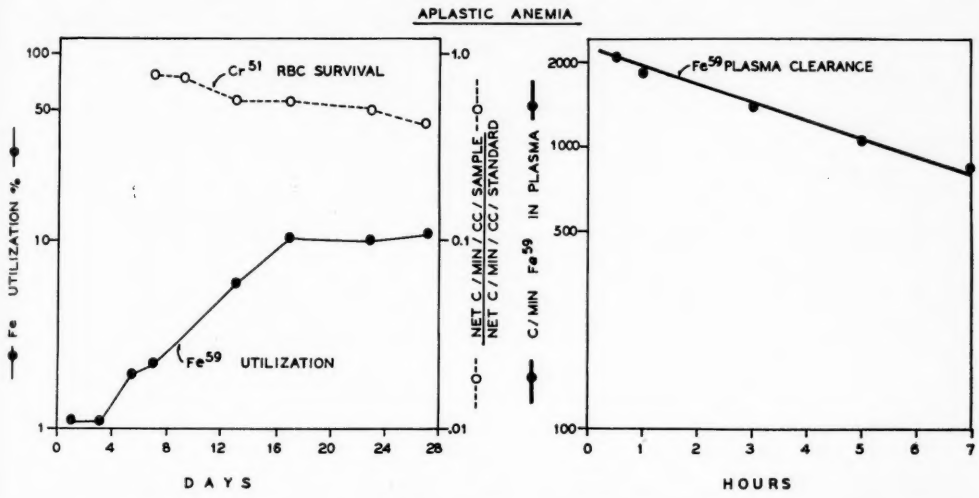

Fig. 3.—Typical curves for radioiron plasma clearance, radioiron utilization by newly formed erythrocytes, and survival time of a mixed population of radiochromium-tagged erythrocytes in a subject with idiopathic aplastic anemia.

SUMMARY

A relatively easy method for the combined determinations of plasma iron clearance, plasma iron turnover, per cent iron utilization, blood volume and erythrocyte survival time has been presented. This is based upon Cr^{51} and Fe^{59} isotope dilution techniques. These quantititive analyses may be done simultaneously in the same individual because the isotopes can be distinguished in the same blood sample by means of a pulse height analyzer. A simple formula has been derived to determine the radioactivity emanating from each isotope.

Data have been presented that establish the values for each of these indices in normal human subjects. These values are similar to those reported in earlier investigations from this and other laboratories in which each of the isotopes was used separately. Minor differences in rates of iron plasma clearance and per cent Fe utilization were possibly due to the higher specific activity of the Fe^{59} used in these studies.

Results from typical cases of hemolytic anemia and aplastic anemia are shown. These indicate the usefulness of the combined procedure for evaluating erythrocyte production and destruction and give the limitations of some of the indices, particularly the Fe^{59}-plasma clearance and the plasma Fe^{59} turnover rate.

We gratefully acknowledge the helpful advice and criticism of Drs. Leon O. Jacobson, George V. LeRoy, and William Bethard, and Mrs. Antreen Pfau in preparing this paper. Mrs. Pearl Avery performed valuable technical assistance.

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METABOLIC STUDIES IN WILSON'S DISEASE USING Cu^{64}

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ALTHOUGH considerable information concerning the metabolic abnormalities in Wilson's disease has been obtained from extensive work carried out in laboratories both in England and America,[1-8] the precise pathogenesis of the disease remains speculative. In an attempt to clarify certain aspects of the problem, normal subjects and patients with Wilson's disease have been given radioactive copper, and a comparative study has been made of its distribution and excretion. A preliminary communication of part of this work concerning the localization of radioactivity in various serum protein components after administration, both orally and intravenously, has already been reported.[9]

MATERIALS AND METHODS

Six patients with Wilson's disease and four normal controls were studied. Radioactive Cu^{64}, either in the form of the nitrate or the acetate, was administered on separate occasions, by both the oral and the intravenous routes. In the present series of experiments no differences were noted in the handling of Cu^{64} whether given in either form. The dose of radioactivity was 0.1 mc., containing 1.25 mg. of copper, when given intravenously, and 1.0 mc., containing 12.5 mg. of copper, when given by mouth. Radioactivity was determined in the serum, urine, and feces at various time intervals after its administration. The material to be measured for radioactivity (2 ml. of serum, 2 ml. of urine, and about 2.5 Gm. wet weight of feces) was placed on a small planchette together with 0.3 ml. of 50 per cent sucrose and 2 to 5 drops of 2 per cent aerosol. The addition of these two latter substances enabled a film of more uniform thickness to be presented to the counting device. The planchettes were heated in an electric oven until the material was dry, particular care being taken to avoid boiling. The radioactivity was measured using an end-window Geiger-Müller counter. Electrophoretic separation of the serum was carried out by the method of zone electrophoresis using a starch-supporting medium.[10]

RESULTS

Disappearance of Radioactive Cu^{64}.—Following the intravenous administration of radioactive copper to normal subjects an extremely rapid initial fall in the serum copper radioactivity was apparent (Fig. 1). One hour after the administration of radioactivity the concentration of Cu^{64} in the serum was reduced from approximately 4,000 CPM to about 300 CPM. In patients with Wilson's disease the initial fall was somewhat slower and 1 hour after the injection of the copper the counts per minute had fallen to approximately 1,200. The striking difference in the shape of the disappearance curves of the radioactive copper in the two groups studied was more apparent in the latter part of the curve (Fig. 1). In normal subjects the radioactivity in the serum was

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still increasing 20 hours after its administration, at which time, due to the short half-life of radioactive copper, the experiment had to be terminated.* In patients with Wilson's disease, however, the radioactivity in the serum continued to fall, although at a considerably slower rate than during the first few hours. About ten hours after the administration of the radioactive copper to patients with Wilson's disease, the serum radioactivity reached a relatively

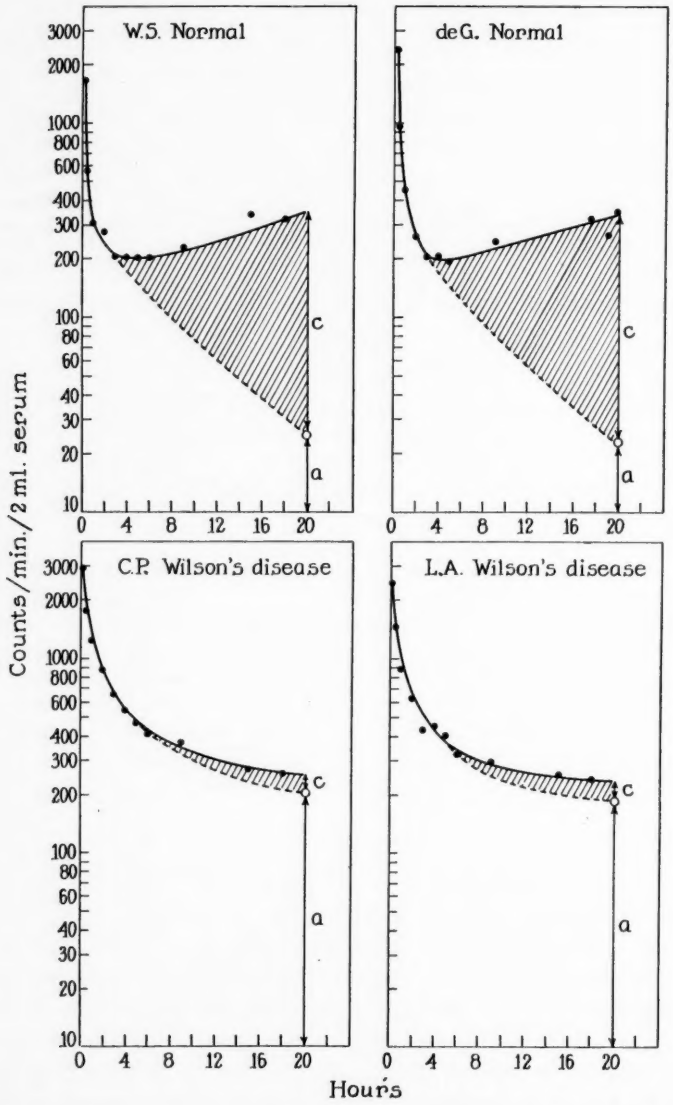

Fig. 1.—Disappearance curves of serum radioactivity following the intravenous administration of 0.1 mc. of Cu^{64}. The dashed line indicates the copper attached to serum albumin. The height "*c*" represents the amount of ceruloplasmin synthesized at 20 hours and the height "*a*" represents the amount of radioactive copper associated with albumin at 20 hours.

*Later experiments using Cu^{64} of higher specific activity permitted continuation of the experiment to 72 hours. Similar curves were obtained and in the normal subjects a plateau of serum radioactivity was reached in approximately 36 hours.

constant level and thereafter declined extremely slowly. In no instance was a secondary rise in the serum radioactivity in patients with Wilson's disease detected.

Radioactive copper was also administered orally to normal subjects and patients with Wilson's disease, and the rise and the fall in radioactivity in the serum were determined. In all normal subjects the initial rise and subsequent fall were followed by a gradual increase in the radioactivity of the serum, which was still increasing when the experiment was discontinued. In subjects with Wilson's disease the initial rise was followed by a slow and steady fall in the radioactivity and no secondary rise was seen, thus simulating the results found when copper was given intravenously. These findings are illustrated in Table I. Analysis of the disappearance curves of radioactivity obtained when the copper was administered orally is complicated by the continued absorption of copper into the blood stream when the serum concentration was already falling.

TABLE I. A COMPARISON OF SERIAL SERUM RADIOACTIVITY MEASUREMENTS IN CONTROL SUBJECTS AND PATIENTS WITH WILSON'S DISEASE FOLLOWING THE ORAL ADMINISTRATION OF 1.0 MC. OF Cu^{64}

	CONTROL				WILSON'S DISEASE				
HOURS	M. G. M.	A. H.	R. F.	A. H.	M. F.	T. M.	V. R.	M. G.	L. A.
½	198						190		
1	323						413		
2	186			749			972	988	
3	132	273	390		479		806		1,350
4	94			755			710	1,405	
5									
6	77		380	624		426	566	1,055	1,1120
7									
8	85						452	784	
9		290		680	370	290			
10	69						444		
11		276			150	276			
12	75		480	750			372	650	960
14									
15				840				534	
16				918				503	
18									680
20			580				150		
22	82								
24	90			998			142	384	
28									
30				1,021			94		
36				1,144				250	420
42				1,610				200	

To supplement the radioactive findings using whole serum, fractionation of the serum was carried out and measurements of radioactivity were made on the various electrophoretically separated protein fractions. In both normal subjects and patients with Wilson's disease the radioactivity was first found to be associated with the serum albumin component. In normal subjects the radioactivity was later found to be incorporated into a protein with an approximate mobility of an $alpha_2$ globulin. In Wilson's disease, however, little or no incorporation of the radioactivity into the $alpha_2$ globulin fraction was

noted, and the radioactivity associated with the albumin was considerably greater than that found in normal subjects at the same time. These findings are illustrated in Fig. 2. Although not shown in this experiment, no radioactivity was associated with the gamma globulin component of serum.

Urinary Excretion.—The excretion of radioactive copper was studied in both groups of subjects, following both oral and intravenous administration of radioactive copper. A striking increase in the urinary excretion of radioactive copper was found in patients with Wilson's disease compared with normal subjects. Following the administration of the copper intravenously the rate of urinary excretion of Cu^{64} was studied in detail in one normal subject and one patient with Wilson's disease for 72 hours. In both patients the amount of Cu^{64} excreted in the urine was greatest during the first 2 hours. In the normal subject the urinary excretion of Cu^{64} per hour became relatively constant about 5 hours after it had been given, whereas, in the patient with Wilson's disease about 12 hours elapsed before a steady rate of excretion of the Cu^{64} became apparent.

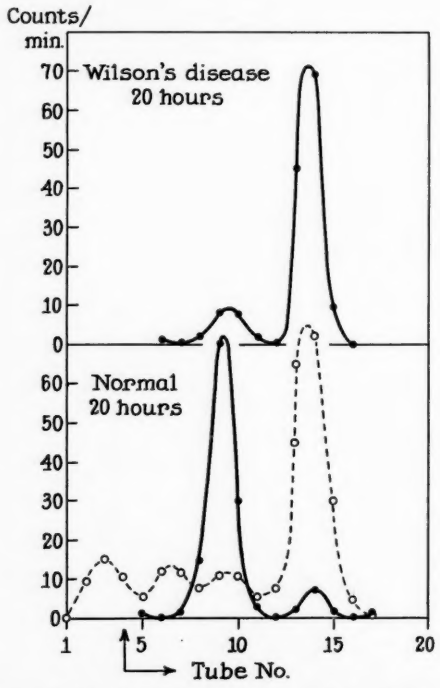

Fig. 2.—A comparison of the electrophoretic separation of serum from a normal subject and a patient with Wilson's disease carried out under similar conditions. The dashed line represents the serum protein curve. The solid lines represent the radioactive measurements in the various protein fractions. The high concentration of Cu^{64} associated with the albumin in the patient with Wilson's disease compared with the small amount in the normal is apparent.

Fecal Excretion.—The fecal excretion of Cu^{64} following oral and intravenous administration was studied in control subjects and in patients with Wilson's disease. Preliminary results had suggested the possibility that following intravenous administration of Cu^{64} less copper was eliminated in the feces of

patients with Wilson's disease than occurred in normal subjects.[9] Further experiments using both oral and intravenous Cu^{64} have now been carried out and the Cu^{64} excreted in the feces compared in the two groups of patients studied. Following intravenous administration of the radioactive copper the control subjects, including a patient with cirrhosis of the liver, excreted a considerably greater quantity of the injected Cu^{64} by the fecal route than did patients with Wilson's disease. Similarly, when the radioactive copper was given by mouth a much greater percentage of the administered copper was recovered in the feces than occurred in patients with Wilson's disease. These results are illustrated in Table II. Because of the very short half-life of Cu^{64} the experiments could not be prolonged beyond 3 to 4 days without the introduction of considerable error; thus the figures illustrated in Table II represent minimum values.

TABLE II. THE EXCRETION OF Cu^{64} IN THE FECES OF CONTROL SUBJECTS AND PATIENTS WITH WILSON'S DISEASE FOLLOWING THE ADMINISTRATION OF RADIOACTIVE COPPER

Cu^{64}	SUBJECT	PER CENT OF THE ADMINISTERED DOSE EXCRETED IN FECES
		(0-72 hours)
Intravenously	Control	12.0
	Control	33.4
	Wilson	0.4
	Wilson	0.9
		(0-96 hours)
Orally	Control	36.4
	Control	43.3
	Wilson	9.2
	Wilson	6.4

DISCUSSION

The extremely rapid early disappearance of radioactive copper from the serum of normal subjects following its intravenous administration and the slightly slower disappearance rate in patients with Wilson's disease is open to several possible interpretations. The half-life of the first accurately measurable component (15 to 30 minutes) in 2 normal subjects was 9 and 10 minutes, respectively, and in patients with Wilson's disease 19 and 20 minutes, respectively. In both groups the rate of disappearance of the radioactive copper is considerably faster than the rate at which Evans blue, another substance, which is attached to albumin, disappears from the serum. Eisenmenger and Slater[11] have calculated that the half-life of the transfer from the plasma of Evans blue bound to albumin is approximately 15.4 hours. The rapid disappearance of radioactive copper from the serum in normal subjects and in patients with Wilson's disease implies a very active uptake by the tissues of the injected Cu^{64} and is analogous to the rapid removal by the liver of Bromsulphalein, which is also bound to albumin. The initial uptake of the Cu^{64} by the tissues is also rapid in Wilson's disease, but slightly less than that found in normal subjects. Since the tissues in Wilson's disease are already saturated with copper it is possible that the decreased uptake is related, at least in part, to this increased saturation. The rapid disappearance

of radioactive copper from the blood stream has also been noted previously by Earl and co-workers.[7] In the latter experiments, however, the initial disappearance rate was faster in the 1 patient with Wilson's disease studied than in 2 normal controls. Subsequently, however, the rate of disappearance of radioactivity appeared to be slower in the patient with Wilson's disease.

The secondary rise in the radioactivity of the serum of normal subjects, which is not found in Wilson's disease, reflects the appearance of the radioactivity in newly synthesized ceruloplasmin. Because ceruloplasmin is deficient in Wilson's disease, the radioactivity of the serum continued to fall. Confirmatory evidence that the rise in radioactivity in normal subjects is due to the incorporation of radioactivity into newly synthesized ceruloplasmin is found in a consideration of the electrophoretic data. It was shown in a previous communication, and confirmed in the present series of experiments, that the radioactive copper following either oral or intravenous administration is first associated with the serum albumin component. The absence of incorporation of radioactivity into the globulin fraction of the serum taken from patients with Wilson's disease is illustrated in Fig. 2. The possibility that the radioactive copper might be associated with the metal-combining globulin is rendered extremely unlikely in view of the findings obtained when radioactive iron is administered and the serum is separated under the same conditions as for the Cu^{64} analyses. Fig. 3 represents an electrophoretic separation of serum from a control subject who had been given radioactive iron. Ninety minutes after its administration the radioactivity was associated with a beta globulin showing a considerably slower mobility than ceruloplasmin.

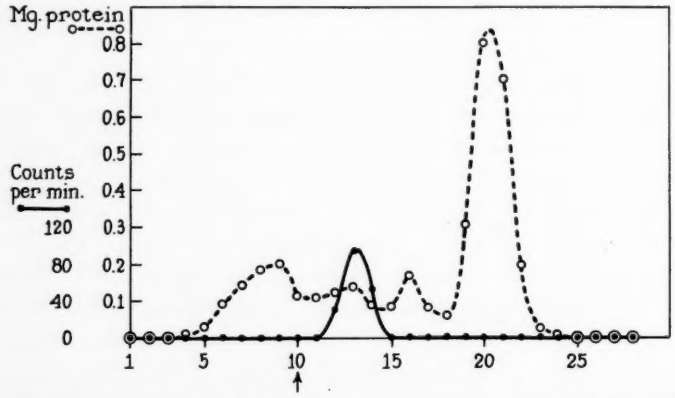

Fig. 3.—Electrophoretic separation of serum from a control subject following oral administration of radioactive iron. The radioactivity is seen in the β_1 globulin region. The dashed line represents the serum protein curve.

Calculations based on the percentage of radioactivity present in the albumin and the ceruloplasmin at 20 hours have enabled a further analysis of the disappearance rate of radioactive copper from serum to be undertaken. The disappearance of the radioactivity from the serum albumin is indicated by the dashed line in Fig. 1. The amount of ceruloplasmin synthesized in both groups studied is represented by the vertical height "*c*" in Fig. 1. A

sizable quantity of the total radioactivity injected can be accounted for in the ceruloplasmin present in the serum at 20 hours. Moreover, since the curve of the radioactivity is still increasing at this time and no allowance can be made for the continuous degradation of ceruloplasmin, substantially more of the administered radioactivity must be incorporated into ceruloplasmin than is apparent from a consideration of the data at 20 hours. In patients with Wilson's disease little or no ceruloplasmin is synthesized and the radioactivity remains associated with the albumin component and declines extremely slowly.

Since it is known that the ceruloplasmin copper is more firmly bound than copper associated with albumin, the amount of copper available for ready deposition into the tissues will depend on the amount of copper attached to the circulating serum albumin and is represented in Fig. 1 by the vertical height "*a*." It is apparent that in Wilson's disease a considerably greater quantity of copper remains bound to albumin than in normal subjects. These findings are in accord with those of Cartwright and associates,[8] who obtained a greater percentage of direct-reacting copper in the serum from patients with Wilson's disease compared with normal subjects, and are also in agreement with the findings of Earl and co-workers.[7] Calculation of the half-life of ceruloplasmin is not possible from a consideration of the disappearance curves of radioactivity since the short half-life of copper precluded the continuation of the experiment until the radioactivity of the newly synthesized ceruloplasmin declined at a steady rate. Since the peak of the ceruloplasmin had not been reached at 20 hours it cannot even be inferred that the rapid uptake of copper in ceruloplasmin indicates a rapid turnover. Moreover, no information is available concerning the nature and the half-life of the precursor of ceruloplasmin, which would be essential in attempting to estimate the half-life of ceruloplasmin.[12] However, in view of the amount of the injected radioactivity associated with the ceruloplasmin at 20 hours, it is tempting to suggest the possibility that future studies, which must await a more stable isotope of copper, will reveal a short half-life for this plasma protein. The possibility is also raised that ceruloplasmin may play some type of precursor role or act as a storage source of copper for other copper proteins of the body.

The increased excretion of radioactive Cu^{64} in the urine of patients with Wilson's disease as compared with that of normal subjects confirmed earlier findings from this laboratory and was also noted by Earl and co-workers.[7] The excretion of the radioactive copper by the kidney in both normal subjects and patients with Wilson's disease probably represents Cu^{64} which has been dissociated from the copper albumin complex during its passage through the kidneys. If this hypothesis is correct the higher rate of excretion of copper in Wilson's disease is in turn related to the persistence of the elevated copper albumin component in this condition. In normal subjects the rapid incorporation of Cu^{64} into the ceruloplasmin molecule diminishes the amount of Cu^{64} which is available for excretion via the kidneys.

The finding of a diminished excretion of Cu^{64} in the feces of patients with Wilson's disease following oral administration could be due to increased

absorption of copper or a decreased re-excretion of copper (or a combination of these two possible mechanisms). However, the observation that following the intravenous administration of Cu^{64} less copper is excreted via the feces suggests that diminished re-excretion may play an important role. Elucidation of the relative importance of increased absorption and decreased excretion of copper in the pathogenesis of the disease is under further study.

SUMMARY

Serum and urinary radioactivity has been studied following the intravenous and oral administration of Cu^{64} to control subjects and patients with Wilson's disease.

Following the intravenous injection of Cu^{64} an extremely rapid fall in serum radioactivity was observed in normal subjects which was subsequently followed by a large increase in serum radioactivity. In patients with Wilson's disease a slightly slower fall in the radioactivity was noted initially and no secondary rise in the serum radioactivity was observed. Electrophoretic separation of the sera at various time intervals revealed that the rise in serum radioactivity in normal subjects was due to its incorporation into ceruloplasmin. Since this protein is deficient in Wilson's disease, no increase in the serum radioactivity was observed.

The radioactivity associated with the serum albumin following oral or intravenous administration was found to be increased and to persist very much longer in patients with Wilson's disease than in control subjects. The persistence of excess administered copper associated with albumin appears to be an important factor in the pathogenesis of the disease.

The total urinary radioactivity excreted in Wilson's disease is greater than in normal subjects. A delayed rise in urinary radioactivity was noted in normal subjects and was not observed in patients with Wilson's disease. However, a diminished fecal excretion of Cu^{64} compared with normal subjects followed both the oral and intravenous administration of Cu^{64}.

We are indebted to Dr. Gerald B. Phillips of the National Institutes of Health, Bethesda, Maryland, for providing the serum sample employed for Fe^{59} localization.

The Cu^{64} was supplied by Abbott Laboratories, Oak Ridge, Tennessee.

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OBSERVATIONS ON AN AGGLUTININ IN HUMAN SERUM FOR PERIODATE-TREATED ERYTHROCYTES

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INTRODUCTION

NUMEROUS investigators have demonstrated that red blood cells can be rendered panagglutinable by a large variety of agents. Thomsen,[1] in 1926, was the first to report that erythrocytes, obtained from suspensions contaminated with certain bacteria, were agglutinable by all normal adult human sera. Soon thereafter, Friedenreich[2, 3] noted that filtrates from cultures of *Vibrio cholera* or certain diphtheroid strains contained a specific enzyme capable of such activity. The panagglutination reaction was referred to as the "Thomsen-Friedenreich phenomenon" or "T agglutination"; the naturally occurring agglutinin being named the "T agglutinin." Interest in this immunologic reaction was revived when Hirst[4, 5] and Burnet and his associates[6-8] reported that viruses belonging to the mumps-influenza group and a specific enzyme obtained from *Vibrio cholera* filtrates (receptor-destroying enzyme—RDE) as well as periodate ions were all capable of rendering red cells panagglutinable. These investigators further demonstrated that such altered erythrocytes were capable of inducing antibodies which would react with the treated red cells, but not with unaltered red cells. Moskowitz and Treffers[9] in this country, and Stewart[10] in England, reported that the "T agglutinin" and the hemagglutinin produced by periodate-treated erythrocytes were serologically distinct. The present investigation was undertaken to study in further detail various properties of the periodate agglutinin ("P" agglutinin).

MATERIALS AND METHODS

Serum specimens were taken in the Baltimore Rh Laboratory from 332 normal individuals—72 males, 90 females, and 170 pregnant females. Neonatal blood was collected by careful needling of an umbilical vessel at delivery, and maternal samples were obtained within twelve hours of birth. Serum specimens were collected from ward patients of the University Hospital with acquired hemolytic anemia, acute rheumatic fever, acute glomerulonephritis, rheumatoid arthritis, and acute disseminated lupus erythematosus.

An isotonic phosphate buffer—pH 7.2—was used as the diluent and washing medium in all experiments. Sodium periodate (Fisher Laboratories) was used as a 0.001 M solution in phosphate buffer.

Periodate-treated cells (PTC) were prepared in the following manner: Equal volumes of a 2 per cent suspension of Group O Rh positive or negative cells and 0.001 M sodium periodate solution were mixed and allowed to stand for one-half hour at room temperature. These cells were washed four times with phosphate buffer using three volumes of buffer for each volume of cells. The PTC were resuspended as a 1 per cent suspension and used immediately. Accurate twofold serial dilutions of serum were prepared with phosphate buffer.

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To 0.15 ml. of the serum dilutions were added 0.15 ml. of the 1 per cent PTC suspension. The mixture was allowed to stand at room temperature for one hour and then centrifuged at 5,000 r.p.m. for 30 to 40 seconds and immediately examined. The highest dilution of serum showing *microscopic* clumping was considered the end point. The titer is expressed as the reciprocal of this serum dilution. Two controls, consisting of PTC in phosphate buffer and the untreated cells in undiluted serum, were performed with each determination. When antibody titers were determined at 4° C. and 37° C., the serum dilutions and PTC suspensions were first equilibrated at these temperatures and then mixed. The temperature was maintained at either 4° C. or 37° C. throughout the entire procedure, and readings were made immediately after centrifugation.

Cold agglutinin titers and Coombs' antibody determinations, both qualitative and quantitative, were determined in the customary fashion.[11]

EXPERIMENTAL RESULTS

1. *Effect of Temperature on Periodate Agglutinin Determinations.*—Periodate agglutinin determinations were carried out at various temperatures: room temperature (21° to 23°C.), 4° C., and 37° C. Serum specimens, obtained from normal individuals, were examined for the presence of cold agglutinins; these agglutinins were removed by absorption if present. The periodate agglutinin titers of representative sera at various temperatures are recorded in Table I. It will be noted that consistently higher titers were obtained for the same serum at lower temperatures. It was also observed that while macrosopic and microscopic end points were identical at 4° C., the latter was usually one to two tubes higher than the former at both room temperature and 37° C.

The periodate agglutinin is thermolabile in that incubation at 65° C. for one hour resulted in the complete denaturation of this antibody. Thermolability alone, however, is not an adequate explanation of the results recorded in Table I, for serum stored under sterile conditions at room temperature and 37° C. for one week exhibited no alteration in the periodate agglutinin titer.

TABLE I. EFFECT OF TEMPERATURE ON PERIODATE HEMAGGLUTININ DETERMINATIONS

SERUM SPECIMEN	1	2	3	4	5	6	7	8	9	10	11	12	13
4° C.	640	320	320	40	320	320	80	320	320	80	640	160	160
R.T.*	320	160	160	10	160	160	20	160	160	80	320	80	80
37° C.	80	80	40	0	80	80	0	80	80	40	160	40	40

*R. T. = Room temperature (21° C. to 23° C.).

2. *Distribution of Periodate Hemagglutinin in Normal Adult Human Serum.*—A total of 332 sera were studied using both Rh_o positive and negative cells as PTC in determining the periodate agglutinin titers. All Rh_o negative sensitized individuals were excluded from this series. The results are plotted in Fig. 1. The normal distribution of the periodate hemagglutinin titers at room temperature was 10 to 640, with 61 per cent of the individuals within the range of 80 to 160. Similar results were obtained with both Rh_o positive and negative cells used as PTC. These titers are slightly higher than those reported by other investigators.[9] This difference is attributed to our practice of determining the end point of agglutination by *microscopic* rather than *macroscopic* methods.

The periodate agglutinin titers were not influenced by the major blood group, sex, or race (Caucasian or Negro) of the individual. No differences were noted between pregnant and nonpregnant females.

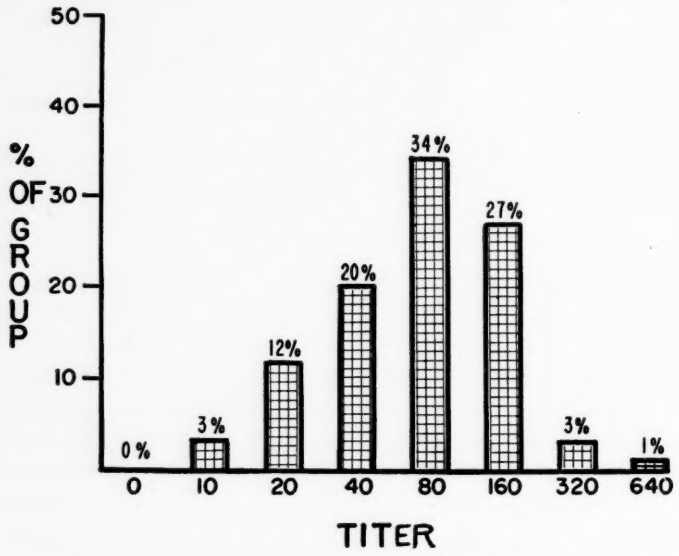

Fig. 1.—Periodate agglutinin titer in 332 normal individuals.

3. *Determination of Periodate Agglutinin Titers in Neonates.*—Sixteen maternal-neonate pairs were studied. All mothers had been delivered uneventfully of full-term healthy infants. Cord blood samples were obtained by needling an umbilical vessel at birth, and maternal specimens were taken within 12 hours of delivery. Rh_0 negative sensitized individuals were excluded from this series, as were patients showing a positive serologic test for syphilis. The results are recorded in Table II. Although maternal blood contained normal periodate agglutinin titers, neonate blood failed to demonstrate the presence of this antibody.

TABLE II. PERIODATE HEMAGGLUTININ TITERS IN NEONATE BLOOD

PAIR NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Maternal titer	80	80	160	160	320	80	80	40	80	160	80	160	160	80	160	40
Neonate titer	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

4. *Relationship of Periodate and Cold Agglutinin.*—Thirty-four serum specimens from ten patients with acquired hemolytic anemia, demonstrating high cold agglutinin titers (48 to 768), were studied to determine the relationship between these agglutinins and periodate hemagglutinins. Periodate and cold agglutinin titers were determined on one aliquot using untreated serum; the other aliquot was divided into two parts and studied in the following manner: On the first part, following the complete selective absorption of periodate agglutinin, cold antibody titers were determined; on the second sample, the reverse procedure was employed, i.e., the cold agglutinins were absorbed

out and the periodate agglutinin titers were then measured. The results of six such studies are recorded in Table III.

TABLE III. THE RELATIONSHIP OF COLD AND PERIODATE HEMAGGLUTININS AS DEMONSTRATED BY DIFFERENTIAL ABSORPTION STUDIES

	ALIQUOT I (UNTREATED)		ALIQUOT II ("P" AGGLUTININ ABSORBED)		ALIQUOT III (COLD ANTIBODIES ABSORBED)	
SPECIMEN	COLD ANTIBODY	P AGGLUTININ	COLD ANTIBODY	P AGGLUTININ	COLD ANTIBODY	P AGGLUTININ
1	768	320	768	0	0	320
2	768	320	768	0	0	320
3	768	320	768	0	0	160
4	48	40	32	0	0	40
5	48	20	48	0	0	20
6	64	80	64	0	0	80

The results indicate that cold agglutinin and periodate agglutinin are apparently serologically separate entities. Since the erythrocyte is altered in some fashion by the periodate treatment, cold agglutinin titrations were performed with both periodate-treated and untreated red blood cells, having first absorbed out the periodate agglutinin from the sera studied. Table IV presents the results obtained in this experiment. The results indicate that periodate ions did not alter that portion of the cell required for the cold agglutinin reaction.

TABLE IV. COLD AGGLUTININ DETERMINATIONS USING NORMAL AND PERIODATE-TREATED ERYTHROCYTES

SPECIMEN	"P" AGGLUTININ TITER	COLD ANTIBODY TITER USING NORMAL CELLS	COLD ANTIBODY TITER USING PTC
1	320	512	512
2	320	384	384
3	160	384	384
4	20	48	32
5	20	32	32
6	80	768	768
7	160	64	64

5. *Relation of Coombs' Antibody and Periodate Agglutinin.*—A 5 per cent suspension of Coombs' positive red blood cells obtained from seven patients with acquired hemolytic anemia was divided into two aliquots, one being treated with 0.001 M sodium periodate and the other serving as a control. Direct quantitative Coombs' tests were run using both types of cells (Table V). The findings indicate that periodate treatment did not alter the Coombs' titers of these cells.

Normal cells treated with 0.001 M sodium periodate were mixed with antihuman globulin rabbit serum, and agglutination was observed in all instances. These titers, however, were within the range of periodate agglutinin values previously reported as occurring normally in rabbit serum.[9, 10]

6. *Effect of Periodate Ions on Adult Human Serum.*—Burnet[12] reported that periodate ions destroyed the viral hemagglutinating inhibitory properties of mucoproteins. The results obtained when serum, treated with 0.001 M

TABLE V. EFFECT OF PERIODATE TREATMENT OF NORMAL AND COOMBS'-POSITIVE ERYTHROCYTES

R.B.C. SPECIMEN	COOMBS' TITER BEFORE PERIODATE TREATMENT	COOMBS' TITER AFTER PERIODATE TREATMENT
Coombs' positive		
1	192	192
2	192	192
3	24	48
4	96	96
5	48	24
6	24	24
7	384	384
Normal cells		
1	0	24
2	0	12
3	0	12
4	0	8

TABLE VI. EFFECT OF PERIODATE TREATMENT OF ADULT HUMAN SERUM AS MEASURED BY PERIODATE HEMAGGLUTININ DETERMINATIONS

SERUM SPECIMEN	NORMAL CELLS VS. PERIODATE SERUM	PTC VS. PERIODATE SERUM	PTC VS. NORMAL SERUM
1	0	160	160
2	0	160	320
3	0	160	80
4	0	40	40
5	0	40	40
6	0	160	160
7	0	80	80
8	0	40	80
9	0	40	40
10	0	160	160

sodium periodate at room temperature for thirty minutes, one hour, or three hours, was titrated against normal and PTC are summarized in Table VI. The activity of the periodate ions added to the serum was stopped prior to the addition of red cells. This was accomplished by adding a measured quantity of a saturated solution of glucose which was shown by previous assay to be sufficient for blocking the activity of the periodate ion concentration used in this experiment. Statistical analysis of these data failed to reveal any significant difference, indicating that periodate ions apparently had no effect on the periodate agglutinin.

7. *Periodate Agglutinin Determinations in Acquired Hemolytic Anemia.*—Serum periodate agglutinin titers were determined in twenty-three cases of acquired hemolytic anemia on or soon after hospitalization. The results are shown in Fig. 2. When compared with the normal distribution curve the most striking feature is the increased range of titers observed. The character of the distribution curve suggests that two sets of cases are involved, one exhibiting normal periodate distribution titers and the other showing abnormally elevated periodate agglutinin levels. Careful analysis, however, of these cases failed to reveal or suggest any known etiological factors which could separate them into two groups.

Serial periodate agglutinin determinations performed on 13 patients whose original titer was 320 or less showed no alteration of titer in relation to clinical improvement, splenectomy, ACTH, or cortisone administration. In

seven patients whose initial periodate agglutinin titers were markedly elevated (above 320), there appeared to be a diminution in titer toward normal levels coincident with clinical improvement. Thus in one patient, within three weeks after hospitalization, the titer decreased from 10,240 to 640, at which time he requested discharge and follow-up examinations were not possible.

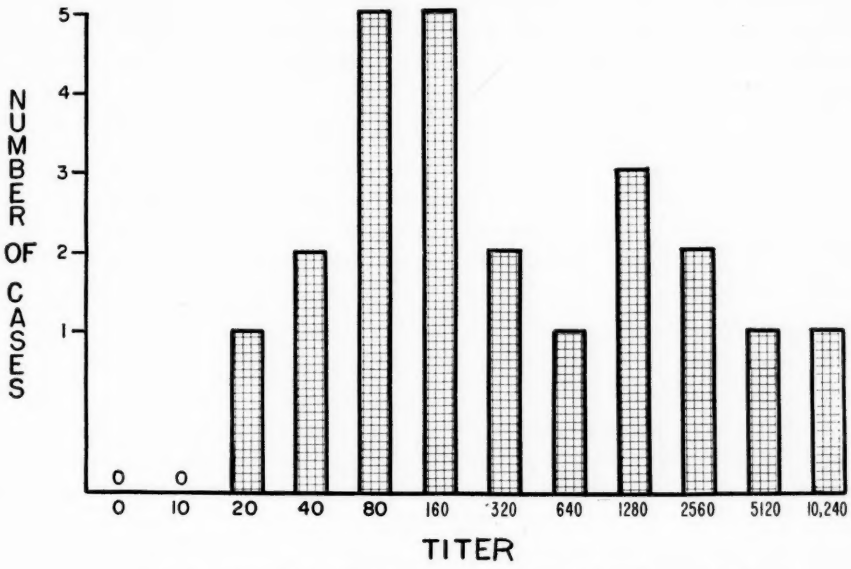

Fig. 2.—Periodate agglutinin titer in acquired hemolytic anemia.

8. *Periodate Agglutinin Titers in Patients With Collagen Vascular Diseases.*—As a preliminary study, periodate agglutinin titers were determined in several patients with collagen vascular diseases. The results, characterized by titers at the upper limits of normal, are recorded in Table VII. When the titers of this series were statistically compared with the normal group, a t value of 3.6 corresponding to a p value <0.01 was obtained. This indicated a significant difference between the two sets of data. The interpretation of this observation is unknown and requires further study. Of particular interest are the low titers of several patients who were receiving ACTH or cortisone.

DISCUSSION

Since the original papers of Thomsen[1] and Friedenreich,[2, 3] numerous bacterial filtrates and viruses[4, 6] have been shown capable of producing panagglutinability of red blood cells. Morton and Pickles[13] and Wheeler and associates[14] have reported that trypsin-treated erythrocytes were rendered panagglutinable. Rosenthal and Schwartz[15] showed this to be an evanescent and spontaneously reversible phenomenon. All the agents thus far reported capable of rendering red cells panagglutinable appear to possess two properties in common: (1) they are organic in nature; and (2) their activity appears to be enzymatic.[16] Both of these characteristics are difficult to associate with the periodate ion.

TABLE VII. "P" HEMAGGLUTININ TITERS IN THE ACUTE PHASE OF VARIOUS COLLAGEN-VASCULAR (HYPERSENSITIVITY) DISEASES

CASES DIAGNOSIS	"P" AGGLUTININ TITER
Acute rheumatic fever	
1	1,280
2	160
3	320
4	320
5	320
6	160
Acute glomerulonephritis	
1	160
2	320
3	160
4	160
5	1,280
Active rheumatoid arthritis	
1	160
2	320
3	160
4	160
5	320
6	160
7	160
Acute lupus erythematosus (disseminated)	
1	320
2	320
3	320
4	640
Periarteritis nodosa	
1	320
Patients receiving ACTH or cortisone	
Active rheumatoid arthritis (1)	10
Active lupus erythematosus (1)	40
Acute rheumatic fever (2)	10

The agglutinin for periodate-treated cells has been shown to be serologically distinct from the "T" agglutinin as well as cold and Coombs' antibodies. Periodate ions apparently do not affect those receptors of the red cell membrane involved in cold or Coombs' antibody agglutination, nor do they remove adsorbed antibody from the cell surface. These observations are in agreement with the view that the erythrocyte surface is composed of multiple antigenic configurations and need not be completely coated in order to produce specific agglutination responses. Brunius[17] has calculated that 30 Forssman antibody molecules are required to sensitize a single cell for hemolysis and the fraction of cell surface covered would be 0.001 per count. Similarly, Heidelberger and associates[18] calculated that 0.04 per cent to 1.6 per cent of sheep red cells' surface occupied by hemolysins was sufficient to cause hemolysis when complement was added.

The periodate agglutinin titer is not influenced by race, sex, or major blood group and occurs in similar amounts in other mammalian species.[9, 10, 16] Its absence from neonate blood is of great interest, since very few antibodies present in the maternal circulation fail to appear in the newborn infant's

blood.[19-21] The absence of the periodate agglutinin from the newborn infant's circulation may be explained by either its inability to cross the placental barrier or its destruction in the neonate. The possibility, however, that altered serum protein characteristics in the newborn infant[22, 23] may preclude its detection cannot be excluded. Human beings have been shown to develop adult titers of the "T" agglutinin by the age of 7.[19] Further studies are indicated to determine if a similar situation exists for the periodate antibody.

The periodate agglutinin is thermolabile, but this property alone is not capable of explaining the higher titers observed at 4° C. than at room temperature or 37° C. Altered physicochemical properties of the serum at varying temperatures may be involved, but further investigation is needed. Periodate is known to affect the mucoprotein content of blood, but when added to serum in our experiment, no alteration in the periodate agglutinin titers could be demonstrated. What role the mucoprotein in the serum and possibly on the red cell surface plays in this agglutination reaction is still to be determined.

The antigenic stimulus responsible for the appearance of this antibody is as yet unknown, although it is highly improbable that periodate ions are responsible. Antibodies for specifically altered erythrocytes may play an important role in the pathogenesis of various disease entities. This hypothesis is particularly attractive in regard to the etiology of acquired hemolytic anemias and conceivably other hypersensitivity states. The diagnosis of acquired hemolytic anemia is based on certain accepted clinical and laboratory observations[24] without knowledge of specific pathogenesis, so that these cases may represent, etiologically, a heterogeneous group. In this series of cases the periodate agglutinin titers varied over a wide range and could not be correlated with the cold or Coombs' antibody levels. Thus one patient with a direct Coombs' titer of over 5,000 had a periodate titer of 40. The increased periodate agglutinin levels observed in the acute phase of collagen vascular diseases and in several patients with acquired hemolytic anemia may be related to certain obscure etiological factors, or may merely reflect an increased sensitivity to antigenic stimuli secondary to the disease state.

SUMMARY

The "P" agglutinin titers of 332 normal individuals were determined. The titers ranged from 10 to 640, with 61 per cent of the group having levels of 80 to 160.

The "P" agglutinin is not present in the neonatal circulation.

The "P" agglutinin is serologically distinct from the "T" agglutinin and cold and Coombs' antibodies.

Periodate treatment of red blood cells does not alter those receptors necessary for cold and Coombs' antibody reactions.

The periodate agglutinin is destroyed by incubation at 65° C. for one hour. This degree of thermolability is not considered an adequate explanation for the higher titers observed at 4° C. as compared to those at room temperature or 37° C.

Periodate treatment of serum failed to alter the "P" agglutinin titer.

Several patients with acquired hemolytic anemia exhibited markedly elevated "P" agglutinin titers not necessarily correlated with a corresponding elevation of the cold or Coombs' antibody titers.

Patients in the acute phase of various collagen-vascular diseases showed significantly higher "P" agglutinin titers when compared to the normal control group. Three patients receiving either cortisone or ACTH (adrenocorticotrophic hormone) had titers at the lower range of normal.

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LABORATORY METHODS

DIRECT METHOD FOR PLATELET COUNTING, WITH A STUDY OF ITS ACCURACY

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HEMATOLOGISTS have long been concerned with methods for platelet counting inasmuch as both the direct and the indirect methods employed are unsatisfactory. In an attempt to overcome the past difficulties, we have developed a new method in which the platelets are well stabilized (fixed) without risk of agglutination, and counted with the ordinary-light microscope, rather than with the phase microscope, as suggested by other authors.[1, 4, 6]

MATERIAL AND METHODS

Material.—All glassware and needles used in collection of the blood and preparation of the platelet suspension in plasma were siliconized, thoroughly dried, and cleaned. The test tubes used for final dilutions for counting, the counting chamber, and the diluting pipettes were not siliconized.

Anticoagulant: The principal constituent in the method is the anticoagulant. A 1.0 per cent solution of Sequestrene-Na_2, which has the property of keeping the platelets in suspension and impeding their agglutination, was employed.[5]

Diluting fluid: Several diluents were tried in our experiments. It was observed early that hypotonic, rather than isotonic, solutions of sodium chloride improved the appearance of the platelets. The compositions of the three diluting fluids were as follows.

Diluent A:	Sodium citrate	2.0 Gm.
	Sodium chloride	0.2 Gm.
	Citric acid	0.1 Gm.
	Merthiolate 1:1,000	1.0 ml.
	Distilled water q.s.	100.0 ml.
Diluent B:	Cocaine	3.0 Gm.
	Sodium chloride	0.2 Gm.
	Distilled water q.s.	100.0 ml.
Diluent C:	Sodium sulfate	3.0 Gm.
	Sodium chloride	0.7 Gm.
	Tween-80	2.0 ml.
	Distilled water q.s.	100.0 ml.

The third dilutent (C) is prepared as follows. The sodium sulfate and sodium chloride are dissolved in 80 ml. distilled water. The solution is heated to 80° C. and then mixed in the 2 ml. of Tween-80. The pipette is cleaned with the hot solution, which becomes opalescent. When the solution regains its transparency at room temperature the final volume is increased to 100 ml.

The data discussed in the present paper were obtained using Diluents A and B; both gave satisfactory results. Diluent B gave better results[4] because cocaine preserves the platelets well and makes them clearly apparent. It is to be noted here that cocaine has the additional property of destroying large numbers of erythrocytes. In the method

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described herein the counts are made on plasma instead of whole blood; hence, there is no need for a substance with such hemolytic activity and also no need of the phase microscope to distinguish between the platelets and the debris resulting from a hemolytic diluent. Unfortunately cocaine is very difficult to get and we were forced to look for a convenient substitute. A more satisfactory solution was devised, using as a base Tween-80, which causes the platelets to stand out more clearly and thus makes it easier to identify stray particles present in the counting chamber.

Method.—

Blood collection: Blood is taken in 10 ml. samples by venous puncture, using a siliconized syringe and needle. The needle must have a caliber of 0.8 to 1.0 mm. (18 to 20 gauge) and a length of 2 to 3 cm. (1 to 1½ inches). The blood sample is slowly mixed in a siliconized vial with 0.1 ml. of the Sequestrene-Na_2 1.0 per cent solution (containing 1.0 mg. of the anticoagulant).

Preparation of a "platelet-rich plasma": The collected blood is placed in a siliconized centrifuge tube and centrifuged at low speed (1,000 r.p.m.) for 3 minutes. With a siliconized Pasteur pipette a little of the floating plasma is transferred to another siliconized tube. This material contains some leukocytes and erythrocytes, but they do not interfere with the counting as they are few in number and are perfectly distinguishable from the platelets.

Counting: After preliminary tests with the usual diluting pipettes it seemed to us that a better method for diluting the "platelet-rich plasma" was to use test tubes. Of the several advantages of this method over that of diluting in counting pipettes, the more important are that it uses a far greater volume of plasma, that it lessens the causes of error during measurement, and that it allows one to vary the titer of the dilution at will.

The final volume must be regulated by the richness of platelets in the plasma. With a little practice the aspect of the plasma itself serves as a guide, as one soon distinguishes, by the suspension turbidity, if the number of platelets is of high, medium, or low order. Lacking practice it is better to make dilutions on the supposition that the number of platelets is normal. Sometimes the platelet concentration is so low that platelets may be counted without dilution, i.e., by placing the plasma directly in the counting chamber. On the other hand, when the number of platelets is too high, a greater dilution is needed.

Dilutions of 1:100 and 1:50 are satisfactory for the normal range. They may be prepared as follows. One or two milliliters of the diluent (for 1:50 and 1:100, respectively) are mixed with the contents of a 0.020 ml. pipette (Sahli) filled with the platelet plasma as described previously. Care should be taken always to wash the pipette several times to avoid letting platelets remain in it. Next, the test tube is shaken for one-half minute, and with a siliconized Pasteur pipette a drop of the suspension is placed in the counting chamber. The suspension is left to settle for about 15 minutes, the time necessary for the sedimentation of the platelets. To prevent evaporation, the chamber should be kept next to a wet piece of cotton under a Petri dish.

The counting is made in an area of 1 mm^2. The platelets appear as round, refractile corpuscles of irregular outline. By turning the micrometric screw it is possible to see the platelets as corpuscles surrounded by a very clear halo. Although debris and dust are not common they are easily distinguished from the platelets.

Calculation of the Number of Blood Platelets.—Once the number of platelets per cubic millimeter of plasma has been determined, the number for the whole blood is calculated according to the blood hematocrit. As the present method is based upon the assumption that by centrifugation 100 per cent of the blood platelets in the plasma may be recovered (see discussion later), to calculate the platelet number per cubic millimeter of blood, one has only to multiply the number of platelets per cubic millimeter of plasma by the percentage of plasma in the blood and divide by 100.

The following experiments were conducted (according to previous statistical delineation) to verify the reproducibility of results and the intrinsic variation of the method for any given plasma (not for whole blood): (1) Study of the platelet distribution in the hemocytometer. (2) Study of the influence of dilution and operator on the results. (3) Study of the error

to verify whether it depends upon the number of platelets and to determine what relation it maintains with that number.

The first two experiments were performed jointly, as follows: (a) Three plasma samples were taken from different persons. (b) Each plasma sample was divided between two operators who performed separately all the subsequent operations. (c) One of the operators was one of the authors (V. M.). The other was unfamiliar with the method but had been adequately briefed on its performance. (d) Each operator worked with four dilutions (1:50, 1:67, 1:100, and 1:200), counting each dilution twice and using two chambers. (e) Each operator registered, for each count, the platelet number in each of 16 fields of $\frac{1}{400}$ mm.^{2} area. (f) The order of preparation of dilutions and countings was chosen with the help of a table of random numbers. The preliminary tests showed that the passage of time up to 5 hours after blood collection did not influence the results.

The third experiment was performed separately. Ten independent counts were made from 3 distinct plasmas, of which the first was poor, the second rich, and the third intermediate in number of platelets.

RESULTS

Distribution of Platelets.—Forty-eight fields were counted (3 plasmas × 4 dilutions × 2 chambers × 2 operators), each with 25 squares, giving a total of 1,200 squares. According to Fisher,[3] the distribution of the blood corpuscles in the hemocytometer follows the Poisson distribution; the mean ($\bar{x}$) and variance (s^2) are estimates of the same parameter, $\mu = \sigma$.

In the case of the trained observer $\bar{x} = 32.54$ and $s^2 = 36.05$, measurements which do not differ significantly. The result is compatible with the hypothesis that the distribution is of the Poisson type. In the case of the untrained technician $\bar{x} = 30.82$ and $s^2 = 20.40$, results which differ significantly, thus excluding the Poisson distribution. The agreement of the results of the trained observer with the Poisson distribution indicates that the hemocytometer is adequate for the counting of platelets. Inasmuch as there are no significant differences between total counts (see later), it is presumed that the unpracticed operator has introduced some bias into the reading of the platelet distribution per square. When questioned on this point the technician revealed a tendency to place in the emptier squares those platelets found on the lines that divide the squares.

Influence of Operator and Dilutions.—Three plasmas were counted, each one by 2 operators, using 4 dilutions and 2 chambers each, and hence giving a factorial experiment with subdivided plots. In this experiment the blocks are the plasma factor dilution (4 levels), operators (2 levels), and finally the experimental units, the 2 chambers, whose counts are multiplied by: 3 in the case of

TABLE I

OBSERVER	DILUTION	I PLASMA	II PLASMA	III PLASMA
Trained (V. M.)	1:50	2,442	9,075	11,073
	1:67	2,168	8,660	9,884
	1:100	2,370	8,748	10,956
	1:200	3,048	11,796	10,428
Untrained (technician)	1:50	2,907	10,440	10,422
	1:67	3,060	10,264	8,900
	1:100	2,898	10,704	10,392
	1:200	2,760	11,244	10,536

Mean = 7,715

1:50 dilution; 4 in the case of 1:67 dilution; 6 in the case of 1:100 dilution; and 12 in the case of 1:200 dilution. The experimental data are shown in Table I.

Analysis of variance of these data shows the results given in Table II.

TABLE II

SOURCE OF VARIATION	D. F.	SUM OF SQUARES	VARIANCE	F	F VALUE AT 5%
Between plasmas	2	301,253,160	150,626,580	–	–
Between operators	1	626,690	626,690	1.04	4.60
Between dilutions	3	3,951,300	1,317,100	2.18	3.34
Interaction operator × dilution	3	689,500	229,832	0.38	3.34
Error	14	8,421,080	601,504	–	–
Total	23	314,841,730	–	–	–

D. F. = Degrees of freedom.
F. = Variance ratio.

It may be concluded that: (a) The untrained operator furnished results that do not differ significantly from those of the trained operator. Thus, the method does not require experience but only adequate briefing. (b) The results obtained from different initial dilutions agree fully, i.e., no platelets are lost with the dilution. This fact permits the choice of the optimum level of dilution for each plasma to be counted in the laboratory.

Study of Error.—Three plasmas of very different platelet numbers were counted. Each plasma underwent ten independent counts. Table III shows the results.

TABLE III

	SAMPLE I	SAMPLE II	SAMPLE III
	663,000	1,105,000	15,500
	690,000	1,153,000	17,300
	790,000	1,301,000	18,300
	728,000	1,311,000	17,250
	650,000	1,213,000	19,170
	698,000	1,257,000	17,600
	765,000	1,235,000	18,500
	726,000	1,290,000	17,500
	693,000	1,225,000	16,700
	761,000	1,214,000	16,700
s	4.66×10^4	6.5×10^4	1.04×10^3
$\bar{x}$	71.64×10^4	123.04×10^4	17.45×10^3
C V%	6.4	5.24	5.7

The coefficient of variability is relatively constant and may be taken, in a general way, as being about 6 per cent. Hence, for a degree of confidence of 95 per cent, we may say that the results will lie between $\bar{x} \pm 11.7$ per cent. That is, when a result of $\bar{x}$ is obtained, 95 per cent of replicate counts of the same plasma will give results between $x - (0.117\ \bar{x})$ and $x + (0.117\ \bar{x})$.

Demonstration of the Recovery of Blood Platelets.—Once we know that the method is reliable, it is necessary to prove that it is consistent, i.e., to demonstrate that all the platelets present in the blood are recovered in the treatment of plasma. To test this, three series of experiments were carried out.

First experiment: A platelet-rich plasma was mixed with a blood so poor that its platelets, added to the plasma, did not influence the counts.

The first sample used was of blood whose plasma showed 5,700 platelets per cubic millimeter as the average of two counts. As the hematocrit of this person showed 57 per cent plasma, it was concluded that there were only 3,250 platelets per cubic millimeter blood. Next, the plasma from an apparently normal person, with a blood type homologous to the first one, was taken. In this plasma the average of the two counts was 681,700 platelets per cubic millimeter.

The blood and plasma were then mixed in equal parts, the mixture centrifuged at 1,000 r.p.m. for three minutes, and the floating plasma separated. Four counts made showed an average of 442,950 platelets per cubic millimeter. The hematocrit of the mixture showed 78.5 per cent plasma.

In this manner it becomes possible to estimate the probable result of platelet count in the mixture:

$$\frac{3{,}250 + 681{,}700}{2} = 342{,}470 \text{ per mm.}^3 \text{ of the mixture.}$$

This result is divided by the percentage of plasma, and multiplied by 100 to determine the platelet number per cubic millimeter of plasma:

$$\frac{342{,}470}{78.5} \times 100 = 436{,}200 \text{ per mm.}^3 \text{ of plasma.}$$

The agreement between the expected and the counted numbers (436,200 and 442,950, respectively) is, evidently, excellent.

This experiment was repeated with greater accuracy using thrombocytopenic blood, in the plasma of which the average of four counts was 553 platelets per cubic millimeter. Notice that this number of platelets can be counted perhaps only by the present method as straight plasma was placed in the chamber. Even so, the four chambers taken together, each with an area of 1.0 mm.^{2}, afforded a total of only 221 platelets. The hematocrit revealed 67 per cent plasma, which gave 371 platelets per cubic millimeter blood (negligible in the mixture). The thrombocytopenic blood was then mixed with a normal plasma which had 466,275 platelets per cubic millimeter as an average of four counts.

Three parts of thrombocytopenic blood were mixed with one part of platelet-rich plasma and the mixture placed in the centrifuge at 1,000 r.p.m. for three minutes.

The floating plasma was separated and eight counts were taken, giving an average of 161,633 platelets per cubic millimeter. The hematocrit of this mixture was 75 per cent plasma. Calculation of the number of platelets in the blood-plasma mixture gave:

$$\frac{(3 \times 371) + 466{,}275}{4} = 116{,}847 \text{ per mm.}^3 \text{ of the mixture.}$$

In accordance with the hematocrit, the number of platelets in the plasma in the mixture should have been:

$$\frac{116{,}847 \times 100}{75} = 154{,}465.$$

This value is extremely close to that actually found (161,633).

Second experiment: This experiment was designed to demonstrate that the proposed slow centrifugation for a short period (maximum of three minutes) recovers 100 per cent of the platelets of the whole blood from the plasma fraction. It is assumed that during centrifugation the following occurs. The erythrocytes, which have a far greater specific weight than the platelets and leukocytes, begin to sediment first. While the erythrocytes are being compressed, they cast forth the plasma, existent between them, together with leukocytes and platelets, especially the latter, which are squeezed outward. Once the hematocrit is attained and the pack of erythrocytes formed, there is no further ascending current of plasma. Only then does the sedimentation of leukocytes and platelets begin.

Ten milliliters of sequestrenated blood was centrifuged at 1,000 r.p.m. for three minutes. The floating plasma was removed and the remainder returned to the centrifuge for three minutes longer. This operation was repeated until no more plasma was obtained. In the present experiment this stage was reached in the fourth centrifugation. Second, an additional 10 ml. of the same blood was centrifuged at 2,500 r.p.m. for one minute and the floating plasma withdrawn. Two independent counts were made of each plasma sample, giving the following averages:

Centrifugation I = 1,369,000 platelets per cubic millimeter.
Centrifugation II = 1,224,000 platelets per cubic millimeter.
Centrifugation III = 657,000 platelets per cubic millimeter.

It is quite evident that in third centrifugation, during which the hematocrit was obtained, there was an accentuated decrease in the number of platelets.

The plasma counts following the centrifugation at high speed (2,500 r.p.m.) gave 1,460,000 platelets per cubic millimeter. Thus it is proved that the speed of centrifugation has no influence on the count when the hematocrit has not been attained.

Third experiment: This experiment was designed to demonstrate that, while the hematocrit is not yet attained, or better, while the pack of erythrocytes is not yet formed, all the platelets present in the original blood are still suspended in the plasma.

Twenty milliliters of sequestrenated blood was placed in each of seven test tubes 60 mm. × 8 mm., care being taken to have the same height of liquid in all tubes (42 mm.). The tubes were then centrifuged for different lengths

TABLE IV

TUBE	HEIGHT OF COLUMN	TIME OF CENTRIFUGATION (MINUTES)	% OF SEDIMENTATION	PLATELETS PER MM.^{3}
1	39	1	7.0	620,000
2	33	2	21.5	620,000
3	29	3	31.0	564,000
4	25	4	41.5	600,000
5	22	6	48.0	443,000
6	21	8	50.0	257,000
7	37	(action of gravity)	12.0	621,000

of time: Number 1 for one minute; Number 2 for two minutes; Number 3 for three minutes; Number 4 for four minutes; Number 5 for six minutes; and Number 6 for eight minutes. Tube Number 7 was not centrifuged, but was left standing for thirty minutes so that sedimentation of erythrocytes in this tube would occur spontaneously due only to action of gravity.

After each centrifugation the height of the erythrocyte column was measured and the plasma transferred to another tube and the counts were made in the usual manner. Two counts were made for each sample. The results are set forth in Table IV.

As may be seen from comparison of the first four and the seventh values, the blood platelet recovery in the plasma is practically 100 per cent. Tubes 5 and 6 show that when sedimentation begins to reach the hematocrit value the platelets are sedimented also.

SUMMARY AND CONCLUSIONS

A method is proposed for the direct counting of blood platelets in plasma. The method is based upon four factors: (a) the use of an anticoagulant which also acts as a preservative for the platelets; (b) the use of smooth, nonwettable surfaces for all preliminary manipulations; (c) direct counts in a hemocytometer chamber; (d) the use of hypotonic sodium chloride diluent with Tween-80 and the common light microscope.

Statistical treatment of the results of this method shows that: (a) the hemocytometer is adequate; (b) no special skill is needed to perform the counts; (c) a wide range of dilutions affords consistent counts; (d) the coefficient of variability is constant and may be evaluated at about 6 per cent; (e) all the platelets present in the blood are recovered in the plasma when centrifugation does not approach hematocrit values.

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A GRAVIMETRIC TECHNIQUE FOR THE DETERMINATION OF PLASMA VOLUMES WITH RADIOIODINATED HUMAN SERUM ALBUMIN

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INTRODUCTION

THE principle of dilution of a known and quantitatively measurable substance has taken precedence in the establishment of simple methods of determining blood and plasma volumes. During the past half century laboratory and clinical investigators have attempted to utilize this principle employing foreign protein,[1, 2] vital dyes,[2, 3] gum acacia, hemoglobin,[4] carbon monoxide,[2, 5-7] and more recently the radioisotopes of iron,[8] phosphorus,[9-12] iodine,[1, 13-15] and chromium.[16-18]

Prior to the availability of radioisotopes, the many methods proposed, with the exception of Evans blue dye, or T-1824,[3] have been technically difficult. Even Evans blue dye has been limited in its clinical application due to the slow disappearance of the dye from the plasma. The clinical application of blood and plasma volume studies is wide in its scope, but depends largely upon the ability of any particular procedure to be repeated at any desired time in order to study blood and plasma volume changes. Accurate estimates of these volume changes make possible definitive therapy in the control of various types of abnormal hemodynamics.

The development of radioiodine-tagged human serum albumin (RISA)* coupled with sensitive instrumentation has made possible the establishment of simple and accurate methods of determining blood and plasma volumes.[13, 14, 19-21] Herein is presented an almost completely gravimetric adaptation of the RISA method of plasma volume estimation utilizing a projection-type analytic balance, a well-type scintillation counter and scaler, and copper sulfate specific gravity standard solutions.

MATERIALS AND METHODS

Preparation of Stock Solution.—Stock solutions were prepared from commercially available RISA. A 1.0 ml. tuberculin syringe was used to transfer approximately 200 μc of RISA under aseptic conditions into a 100 ml. ampule of sterile pyrogen-free normal saline solution. The syringe was flushed five times with saline solution before the needle was withdrawn from the ampule. Stock solutions were refrigerated to inhibit dissociation of the iodine and albumin.

The specific activity of each stock solution was determined gravimetrically. Disposable screw-capped vials 14 mm. in diameter by 50 mm. in length were used for this determination. Each vial was weighed to the nearest 0.0001 gram before and after the addition of one drop of stock solution to the vial from a 22-gauge needle attached to a

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2 ml. Luer Lok syringe. Two or more such vials were prepared and the solution in each vial was diluted with distilled water to approximately 1.0 ml. The vial was then placed into a well-type scintillation counter containing a 1¾ inches diameter by 2 inches long thallium-activated sodium iodide crystal and photomultiplier tube having a standard preset time-controlled scaler.

The specific activity, S, of the stock solution in counts per minute per milliliter can be calculated as follows: $S = A/V$ where A is the activity of the drop in cpm, and V is the volume of the drop calculated from its mass and specific gravity. A value of the specific activity can be determined from each vial. The several values obtained are averaged to give a value for the specific activity which should have a probable error of about 1 per cent or less. These standards may be used until the stock solution from which they have been prepared is exhausted. A series of tests of the stock solution activity during an eight-day storage period showed no significant loss of iodine^{131} due to adsorption of the activity onto the walls of the glass container or to the dissociation of iodine^{131} from the albumin.

Procedure for Determining Plasma Volume.—A 20 ml. Luer Lok syringe was used to withdraw 20 μc of RISA stock solution from the ampule, all air being discharged into the ampule to prevent spillage of the RISA solution. With the patient in the recumbent position, the stock solution was injected slowly into the antecubital vein. Prior to the removal of the needle, the syringe was flushed three times with about 5 ml. of blood.

Samples were withdrawn from the opposite arm, if possible, or from a vein other than the one used for injection. At 15 and 20 minutes postinjection approximately 5 ml. of whole blood was withdrawn and immediately placed into marked heparin tubes. These tubes were inverted twenty times to ensure complete mixing of the heparin and uniform dispersion of all the solid blood elements. A commercially available Pasteur-type disposable glass pipette was used to withdraw approximately 1.0 ml. of heparinized blood. This blood was placed into a previously weighed and clearly marked vial and the weight of blood determined by the use of the automatic balance. The specific gravity was determined in copper sulfate solutions as described by Simmons and Gentzkow.[22] The vial was then placed in the well-type scintillation counter and its specific activity was determined. It is desirable that each reading comprise at least 10,000 counts. A 20 μc dose will result in about 4,000 to 5,000 cpm per milliliter of whole blood. Several 3-minute readings were taken on each whole blood sample to obtain a reasonable degree of statistical accuracy. The remainder of the blood in the heparin tubes was placed into separate centrifuge tubes and these were spun for 30 minutes at a minimum of 2,000 g of centrifugal acceleration.[18]

Plasma samples of approximately 1.0 ml. were obtained from the centrifuge tubes using Pasteur pipettes, and were placed into previously weighed vials from which the specific activity per milliliter of plasma was determined. The specific gravity of the plasma was also determined with standard copper sulfate solutions. A 20 μc dose, with the instrumentation described, will result in 9,000 to 11,000 cpm per milliliter of plasma. An average value was obtained by taking three readings of two minutes each on each plasma sample.

Calculation of Plasma Volume.—Values of the plasma volume, V_P, were computed *directly* from the counts per minute on plasma samples from the formula: $V_P = \frac{A_T}{S_P}$ where A_T is the total activity in cpm injected and is the product of the dose injected (in milliliters) times the specific activity of the stock solution, and where S_P is the specific activity of the plasma sample computed in the same manner as described for the stock solution. The subscript P refers to plasma.

An isotope venous hematocrit was calculated from the average values of the specific activities of blood and plasma. This venous hematocrit is determined from the following formula: Hematocrit (per cent) $= 100\left(1 - \frac{S_B}{S_P}\right)$ where S_B and S_P represent the specific activities of whole blood and plasma samples, respectively.

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RESULTS

Plasma volume studies have been performed on twenty-five normal adult males, twenty-six normal adult females, and twenty-one patients including patients with severe anemia, polycythemia, multiple myeloma, nephritis, hypertension, and elderly preoperative individuals. The average value obtained for the healthy control subjects led to the establishment of a provisional value of 42 ml. of plasma per kilogram of body weight for both males and females. The average values of the isotope venous hematocrits for the normal males and females were determined to be 44.8 per cent and 42.1 per cent, respectively.

DISCUSSION

The purpose of this project was to establish a simple but accurate gravimetric method of plasma volume determination which will reduce the human error of volumetric aliquoting to an absolute minimum. Common experience among laboratory and research personnel is that volumetric measurements are quantitatively inaccurate even with carefully calibrated micropipettes in the hands of experienced technicians. Small errors in volumetric pipetting result in large final errors in many cases, and give clinically unacceptable results with dilutions in the body ranging from 250 to 300 times the tracer dose of RISA.

The projection-type analytic balance eliminates the error in volumetric pipetting and has helped to increase the accuracy of plasma volume determination. In these studies a Sartorius Selecta Rapid 200 gram projection analytic balance was used exclusively. To determine the specific activities for whole blood and plasma, as well as the preparation of standard solution vials, it is necessary to weigh each of six vials two separate times, making a total of twelve weighings, accurate to 0.0001 Gm. With the automatic projection balance each of these weighings takes from 20 to 40 seconds, while with a conventional double-pan balance, a much longer time would be required. This rapid and accurate method of weighing coupled with simple estimations of blood and plasma specific gravity makes it possible to convert weights to volumes without the necessity of volumetric manipulations. Studies by Burton, Waldo, and Zipf[23] showed that the copper sulfate method is dependably accurate to within 0.0003 Gm. per milliliter, and, therefore, may be used in these volume studies without introducing any significant error.

A series of studies was performed using 0.5 ml. to 1.5 ml. amounts of a dilute solution of RISA with the well-type scintillation probe and scaler. The results confirmed the manufacturer's statement that this instrument is insensitive to volume changes under 2 ml., i.e., that the geometry of the sample being counted does not change appreciably with respect to the crystal and that the specific activities are constant from 0.1 to 2.0 ml. It is recommended that volumes be limited to approximately 1.0 ml. although deviations as large as 0.5 ml. will not produce significant errors.

The total error of plasma volume determinations results from three principal sources: (1) the volumetric aliquoting of the stock solution, (2)

statistical errors in counting, and (3) errors in gravimetric determinations. The aliquoting of the stock solution is the single critical volumetric measurement in the entire procedure. This consists of withdrawing 10 to 20 ml. of stock solution containing approximately 20 μc of RISA, and injecting this solution into the vein of the patient. The over-all error introduced by this procedure will not exceed 1 to 2 per cent, if done with reasonable care. Statistical variation increased as the square root of the total number of counts; therefore, probable errors in counting will not exceed 1 per cent in readings of 10,000 counts or more. In weighings of samples of approximately 1 ml., errors are not greater than 0.02 per cent, while probable errors in weighing the drops of the standard solution are of the order of 0.3 per cent. The maximum probable error of a complete determination is less than 5 per cent, and is likely to be of the order of 2 to 3 per cent since the three primary sources of error may partially compensate for one another.

The 20 μc dose was used to establish the technique and provide a sufficient degree of accuracy for routine clinical use. This dosage of 20 μc of I^{131} will permit five or more tests a week without exceeding the maximum total body radiation limit set by health physics officials. It is estimated,[24] for example, that a 50-kilogram individual would receive 60 milliroentgens, while a 80-kilogram subject would receive approximately 36 milliroentgens during the decay of 20 μc of RISA over a period of seven days. This relatively large dosage limits the number of tests that can be made per week; however, somewhat smaller amounts of activity could be used at the sacrifice of the time required to count the samples or the precision of the determination.

During these studies, samples were withdrawn at 15 and 20 minutes postinjection. These time intervals were used because Evans blue dye plasma volumes were being run simultaneously and 15 minutes is the optimum time recommended for the dye test. Work reported by MacIntyre[15] and Zankel[25] indicates that in individuals with fairly good circulation, complete equilibrium of distribution of the RISA may occur in two to three minutes. Both of these groups have published charts showing leveling of activity peaks after 120 seconds in studying peripheral vascular flow. It is possible, therefore, that later studies may show the feasibility of five- or ten-minute mixing times rather than the fifteen- and twenty-minute intervals used in this work.

SUMMARY

An almost complete gravimetric method of plasma volume determination is presented, including a description of the basic equipment, as well as the explanation of each step in the procedure.

The rationale for the use of the gravimetric method, the use of a copper sulfate specific gravity standard solution, and the computation of the isotope venous hematocrit are discussed. A provisional normal value tabulated in milliliters per kilogram of body weight is presented. This method of plasma volume estimation offers speed, accuracy, and simplicity.

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THE DIRECT MICRODETERMINATION OF CALCIUM IN THE URINE BY NEPHELOMETRY

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THE turbidimetric method of Saifer and Clark[1] for water analysis has been modified to permit the direct nephelometric determination of calcium in urine. The calcium is estimated as a stabilized colloidal suspension of the oleate in ammoniacal solution. The method is comparable in precision to those involving ashing and oxalate precipitation, requires a maximum of 0.1 ml. of urine, and entails less than one hour of time for several samples.

METHOD

Reagents.—(1) Potassium oleate reagent: This reagent is prepared by the procedure of Romeo and Gambordella.[2] Approximately 16.5 ml. of oleic acid is shaken with 10 ml. of water containing 3.2 Gm. of potassium hydroxide. The emulsion is transferred to a flask with the aid of 100 ml. of 70 per cent alcohol, refluxed for one hour, and made to 500 ml. with water. (2) Sodium lauryl sulfate: 10 per cent aqueous solution of Duponol. (3) Duponol-oleate reagent: Five parts of potassium oleate reagent are mixed with two parts of 10 per cent Duponol. This solution is stable but must be kept clear by filtration. (4) Ammonium hydroxide, 37 per cent: 37 ml. concentrated ammonium hydroxide made to 100 ml. with water. (5) Calcium standard: With proper precautions taken to avoid spattering, 250.2 mg. of Iceland spar (calcium carbonate) is dissolved in 10 ml. of concentrated HCl and diluted to 100 ml. with water. One milliliter of this stock standard diluted to 100 ml. with water provides a working standard containing 10 γ of calcium per milliliter.

Procedure.—The total urine sample is shaken with 1 ml. of concentrated HCl per 100 ml. of volume to ensure complete solution of the calcium. This is followed by filtration of a small aliquot through a fine fritted glass funnel. Dilution of the urine is necessary at this point if the calcium content is high.

One-tenth milliliter of the urine sample is transferred by means of a pipette or a calibrated syringe into 4.5 ml. of water in a cuvette. Three drops of 37 per cent ammonium hydroxide are added to each sample and the solutions well mixed. Four-tenths milliliter of Duponol-oleate reagent is then added followed *immediately* by thorough mixing. The unknowns are read against the standards at the end of one-half hour. The two standards each contain 10 γ of calcium and are prepared similarly to the unknowns with due attention being paid to keeping the final volumes identical. A reagent blank is also necessary.

The nephelometer is used as a null point instrument. The scale is set to zero with the blank in place and set to 100 on the basis of the algebraic mean of the two standards. The calcium contents of the unknowns are calculated from the formula of the regression line established previously from a standard curve for the individual instrument. If an unknown has too high a calcium content the standards may be set to the scale reading representing the mid-point of the regression line and the calculated values multiplied by 2 without the introduction of undue error.

EXPERIMENTAL

Statistics of the Standard Curve.—In Fig. 1 are shown two representative standard curves. One curve (solid line) was established on the basis of four

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points in triplicate whereas the second (broken line) contained five points in duplicate. The respective regression equations are

$$Y = -3.45 + 10.31\ X$$
$$Y = -4.66 + 10.43\ X$$

The two curves are statistically similar with respect to slope and intercept. The intercepts are real, as would be anticipated from the observation that micelle formation in soap solutions is absent below a critical concentration.[3]

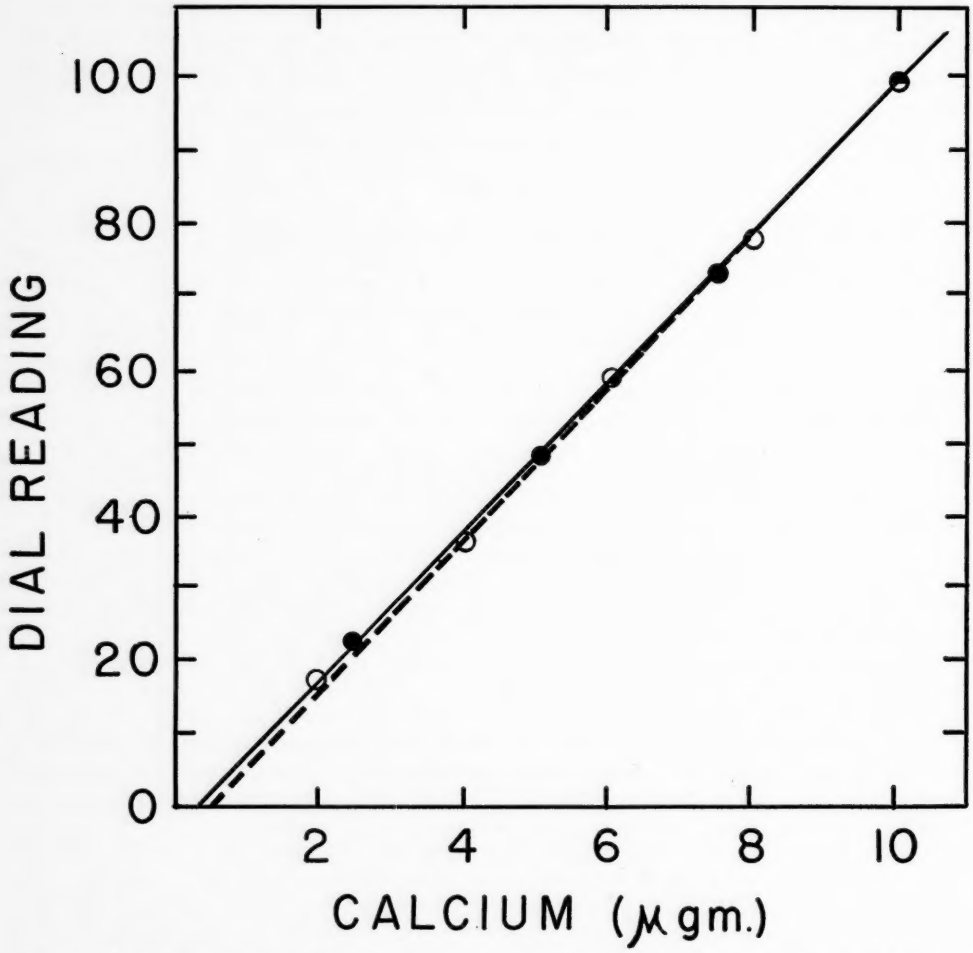

Fig. 1.—Two representative standard curves. Solid line based on four points in triplicate; broken line based on five points in duplicate.

Numerous subsequent standard curves have been made over the past two years by six different operators, and none of these has differed statistically from the two described here.

Comparison With Other Methods.—Twenty-two different 24-hour collections of urine were analyzed by the present method and by methods involving combustion followed by precipitation of the calcium as the oxalate with sub-

sequent permanganate titration. Nine of the samples were dry-ashed in a muffle furnace according to the procedure of Tisdall and Kramer.[4] The remainder were oxidized with ammonium persulfate in acid solution as recommended by Shohl and Pedley.[5]

Another series of 13 urine specimens was analyzed by the present method and by a modification of that of Shohl and Pedley. In this modification, the quantity of urine sample and of reagents was reduced to one-fiftieth that originally proposed. The oxidation, precipitation, and color development were carried out in a scrupulously clean 15-ml. centrifuge tube. The oxalate was determined by the colorimetric procedure of Loureiro and Janz[6] with the exception that daily standards, rather than a standard curve, were utilized since it was found that the curve, although always linear, varied considerably in slope from day to day.

TABLE I. COMPARISON OF PRESENT METHOD WITH COMBUSTION METHODS FOR URINE CALCIUM

METHOD OF COMBUSTION	NUMBER OF SAMPLES	URINE Ca (MG./24 HR.)	DIFFERENCE BETWEEN PRESENT AND COMBUSTION METHOD			
			MEAN	VARIANCE	S.E.*	t†
Dry (Tisdall and Kramer)	9	30 to 145	0	31.75	1.88	0
Wet (Shohl and Pedley)	13	9 to 170	−1.49	32.17	1.57	0.947
Wet (Modified Shohl and Pedley)	13	58 to 285	−2.50	39.33	1.48	1.30

*Standard error of mean.
†See Reference 7.

Comparison of the results obtained from triplicate analyses by all methods is summarized in Table I. There is no statistically significant difference between the data obtained by the present direct procedure and those from methods involving combustion.

TABLE II. MORE DETAILED ANALYSIS OF THE COMPARISON BETWEEN THE PRESENT METHOD AND THAT OF SHOHL AND PEDLEY

SAMPLE NUMBER	URINE Ca IN MG./24 HR.	
	PRESENT METHOD	METHOD OF SHOHL AND PEDLEY
1	9.2	8.5
2	9.9	12.0
3	29.7	30.0
4	41.3	43.7
5	47.7	52.3
6	91.7	93.7
7	95.3	96.0
8	126	115
9	115	121
10	123	126
11	138	134
12	136	137
13	156	170*
Variance†	5.96	10.92

*One titration value obviously in error, but insufficient sample to repeat analysis.
†Error variance within triplicates.

Individual values obtained from the second group of Table I are presented in Table II. The much larger variance of the combustion method may be attributable to the diverse analytic steps involved, which include calcium oxalate precipitation, filtering, triple washing, precipitate transference, and titration with permanganate. If so, any discrepancies which appear in Table II might well be a reflection of the intricacies of the combustion method rather than evidence of an inherent defect in the proposed direct method with its lower variance.

Optimal Reagent Concentrations.—Standards containing 10 γ of calcium were treated with Duponol-potassium oleate solutions of varying composition. The levels of potassium oleate tested were 0.1, 0.3, 0.5, and 0.7 ml. with Duponol concentrations ranging from 0 to 875 mg. per 100 ml. in seven graded steps. The amount of Duponol used in the present method was found to produce the highest colloid formation that was relatively independent of potassium oleate concentration over a wide range.

Influence of Time and Temperature.—The time of reading the 90° Tyndall light is not critical; no appreciable relative change has been noted in the 25- to 60-minute interval following addition of the Duponol-oleate reagent.

While standard curves made at different laboratory temperatures did not vary significantly in slope, the heat from the exciter lamp in the nephelometer was sufficient to produce a 6° C. increase in temperature within 5 minutes in a sample left in the cuvette well. This was accompanied by a 6 per cent decrease in the 90° Tyndall light, the loss being appreciable at the end of one minute.

Interfering Substances.—The following materials did not interfere when present in concentrations one might expect in normal or in pathologic urine[8, 9]: sodium, potassium, magnesium, chloride, phosphate, sulfate, urea, and uric acid. The same held for serum albumin at a level of 4 mg. per sample and for citrate when present at a concentration 1,000 times that of calcium. Cupric nitrate has no demonstrable effect on the reaction and has been utilized to preferentially displace calcium in chelation with ethylenediaminetetraacetic acid. Previous oxidation of the urine sample with persulfate or perhydrol also resulted in no detectable interference.

Both formaldehyde and trichloroacetic acid caused a decrease in the formation of colloid. When applied to the direct analysis of blood serum, the method yielded results which were approximately one-sixth too low. Attempts to characterize the nature of the interference have failed.

Instrumentation.—A Coleman Electronic Photofluorometer, Model 12B, was employed in the investigation of optimal reagent concentrations. This instrument was modified for nephelometry by elimination of the replaceable filters and masking of the reflecting mirror with rubber tape. Since 90° Tyndall light is plane polarized, a polaroid filter was placed in the path of the incident beam to minimize stray light. Calibrated Pyrex test tubes served as cuvettes.

All subsequent studies were conducted with the use of a Coleman Model 9 Nepho-Colorimeter. Employment of a polaroid filter was continued but the immersion well was not utilized. Cuvettes were of the square type, 13 by 13 by

100 mm., and were calibrated with colloidal starch solution. Of 44 cuvettes made from the same lot of glass, 25 were obtained with a standard deviation of ± 0.54 per cent.

DISCUSSION

The present method is a conveniently flexible one. Linearity is maintained when the standard, set to a scale reading of 100, contains from 5 to 20 γ of calcium. The sensitivity of the method may be greatly increased by elimination of the polaroid filter, but at the expense of precision.

Apart from the standard, meticulous care is not necessary in the preparation of the reagents. However, one should exercise precautions common to all nephelometric procedures, such as maintaining light transmitting surfaces free from dirt and scratches and removing suspended materials from reagents by filtration through fine fritted glass funnels.

SUMMARY

A micromethod for the direct nephelometric determination of calcium in urine is described. Calcium is estimated as a stabilized suspension of the oleate in ammoniacal solution. A maximum of 0.1 ml. of urine is required and several samples may be analyzed within one hour.

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USE OF CHICK EMBRYO AS MEDIUM FOR RAPID CULTIVATION OF TUBERCLE BACILLI

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AN ATTEMPT is made to determine that chick embryos offer a medium for the more rapid identification and isolation of *Myco. tuberculosis* than can be obtained with other animal and artificial cultural techniques, without loss of the efficiency of these latter methods. Procedures utilized in this study are not new or original, although modifications of other systems have been appended.[1-4]

Six- or seven-day-old chick embryos are candled to determine location of the embryo and air cell. A small (0.5 cm.) scratch is drilled into the shell above the cell. Following inoculation through it, the hole is sealed with colloidin and the egg incubated in a standard bacteriologic incubator at 37° C. for from four to six days. Eggs are harvested between the fourth and sixth days of incubation if the embryo is viable. Death of the embryo during the first 48 to 72 hours occurs in about 10 per cent of the eggs and negates any interpretation. Approximately 15 per cent of the embryos die between the third and sixth days of incubation. These are harvested promptly, smeared, and stained for tubercle bacilli and subcultured.

Tubercle bacilli ($H_{37}R_V$) are grown in Tween-albumin medium for five or six days,[5] at which time each 1 ml. of medium generally contains ±10,000,000 viable organisms, as determined by plate count. Five to seven dilutions are made from 1 ml. of the fluid medium containing tubercle bacilli 1:100 through 1:100,000,000. Six eggs are inoculated with 0.5 to 1 ml. of each dilution.

After four to six days' incubation, the yolk is examined. Contents of the whole egg are poured into a sterile Petri dish. Membranes of the yolk sac are teased away as completely as practicable and 2 or 3 ml. of the liquid yolk transferred to a sterile tube. One milliliter of this untreated yolk material from each egg is planted on ATS medium.[6] Ten to fifteen milliliters of sterile distilled water are added to the residual liquid yolk and the tube is shaken vigorously until there is a smooth emulsion. To this is added 2 ml. of melted crystalline phenol (to reduce hazard of contamination) and the whole is allowed to stand at room temperature for from one to two hours. This permits separation of the fatty material in which the tubercle bacilli are concentrated. A small amount of the fat layer is transferred to a slide, stained with carbol fuchsin, and counterstained with light green.

Sixteen control series (average six dilutions of tubercle bacilli in Tween-albumin medium in each series) were studied; a total of over 1,100 eggs were thus examined.

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In most instances tubercle bacilli were recovered from yolks upon subculture on ATS medium, and these cultures were uniformly positive on or before the tenth day of growth on subculture. Only two series (twelve eggs) were inoculated with a 1:100,000,000 dilution of tubercle bacilli; both were positive by the tenth day of growth in subculture. Inoculation of greater numbers of *Myco. tuberculosis* into yolk sacs (in dilutions of 1:100 to 1:1,000,000) resulted in positive subcultures on ATS medium frequently recognized by the seventh or eighth day.

Smears of yolks inoculated with approximately 1,000 organisms were observed to be positive easily and without hazard of a false positive or false negative interpretation. Inoculation of from 10 to 100 organisms (dilutions of 1:1,000,000 to 1:10,000,000) into the yolk sacs resulted, after six days' incubation, in material which was somewhat difficult to interpret. Generally a single acid-fast bacillus could be found in individual smears of yolks from two or three of the eggs, rarely in all six in the series. Interpretation of these smears caused some concern before final confirmation by subcultures was secured, but in every series if all six smears from the six eggs were scanned carefully, the smears were positive, at least in retrospect. Difficulties were encountered in reading smears with few organisms because of the frequency of artifacts staining dark (but not red), because organisms were generally single rather than in clumps, and because the young forms failed to have typical morphology. Despite the difficulties, however, final evaluation of smears made from each series of six eggs as a whole was consistent and satisfactory.

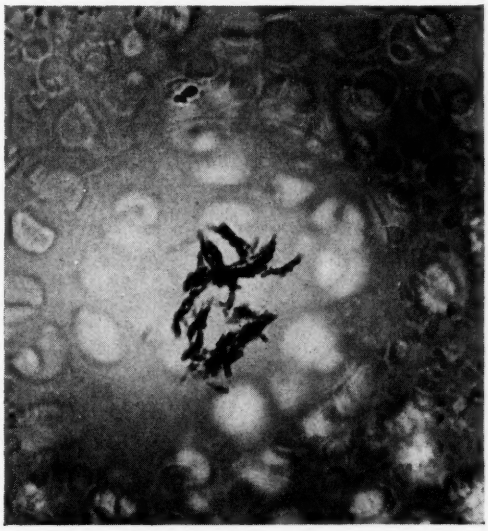

Fig. 1.—Tubercle bacilli seen on smear of yolk sac after six days' incubation. Dark-staining material is acid-fast. Original inoculum into chick embryo was approximately 100 organisms (1:100,000 dilution of tubercle bacilli in Tween-albumin medium).

Fig. 1 is a photograph (magnification ×1,350) of a smear showing tubercle bacilli taken from yolks of chick embryos. The organisms are easily recognized, are typically acid-fast, and in this instance are clumped. This smear was made six days following the inoculation into a chick embryo of a 1:100,000 dilution

of Tween-albumin medium containing a five-day growth of tubercle bacilli (the inoculum probably contained ±100 organisms).

DISCUSSION AND SUMMARY

The yolk sac of a six- or seven-day-old chick embryo is used as the initial culture medium for the rapid identification and isolation of human strain tubercle bacilli. Inoculation of approximately 1,000 organisms into the yolk sac is sufficient to yield, after six days' incubation, positive smears. Subculture upon ATS medium becomes positive in from seven to ten days with this number of organisms. Recognition of tubercle bacilli on smears made from yolk sacs inoculated six days before with from 10 to 100 organisms is difficult, but in any series of six eggs, at least two smears contain a few organisms. Subcultures on ATS medium are positive, however, on the tenth day with the original inoculum containing no more than ten organisms (longest total time for isolation in this report—sixteen days).

It is generally believed that a concentrated specimen of human sputum or other body fluid must contain in excess of 50,000 tubercle bacilli per milliliter if it is to be positive on smear.[7, 8] Identification of lesser numbers of organisms requires culture or animal inoculation, and recognition of tubercle bacilli from them generally requires from three to five weeks. It is our distinct impression that tubercle bacilli can be identified more rapidly with the use of chick embryos from original smear and from subculture. It is our belief, less well documented with present experience, that positive cultures are attained with fewer organisms in the initial inoculum than can be achieved with the use of either solid medium or guinea pigs directly. Thus, increased speed of identification, without loss of efficiency or accuracy, has been effected. On the other hand, the technique has clear deficiencies. Recognition of acid-fast bacilli in a background of fat, precipitated protein, and no cells is difficult. There is no saving of time in the method; technically, it may even take longer to prepare and interpret results. The inoculation and harvesting of the infected material are not arduous, but they do require that new procedures be learned. These defects can be overlooked if, in fact, isolation and identification of tubercle bacilli can be secured at least one and usually two or more weeks sooner than can be anticipated with older culture and animal methods.

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PROGRESS REPORT

PRIMARY ALDOSTERONISM

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ON OCT. 29, 1953, we first described a new clinical syndrome which we named primary aldosteronism. In an addendum to the published report[1] we indicated that at operation, which had been planned as a bilateral adrenalectomy, a cortical adenoma arising from the right adrenal gland was found. The left adrenal gland, therefore, was not removed. A right adrenalectomy which included the tumor was performed by Dr. William C. Baum of our surgical division on Dec. 10, 1954.

The purpose of the present brief report is to indicate that all of the chemical deviations which make up this fascinating metabolic abnormality became normal within ten days following removal of the adrenal tumor* and have remained so to date (three months). Rigid metabolic studies have continued from the time of the original report to the present and thus include the entire preoperative, operative, and postoperative periods (Oct. 26, 1954, to March 10, 1955). Preoperative data between October 26 and December 10 (the day of operation) did not differ significantly from those already reported (April to October, 1954) except for a greater degree of negative potassium balance than was observed in the earlier period.

Although bilateral adrenalectomy was contemplated, it was decided not to employ preoperative preparation with steroids. Large muscle biopsies were to be obtained at the time of surgery for determination of their content of sodium and potassium. Since these values could be changed by such preoperative medication, intravenous hydrocortisone was begun in the operating room immediately after the specimens of muscle had been obtained. In addition, biopsies of both kidneys and of the left adrenal gland were taken.

SUMMARY OF RESULTS

DEC. 10, 1954 (OPERATION) TO MARCH 10, 1955

1. *Surgical Specimens.*—

a. *Adrenal tumor:* This was almost round, measured 4 cm. in diameter, and replaced four-fifths of the right adrenal gland. It was deep yellow on cut section. Microscopically it was a well-encapsulated adrenal cortical adenoma, the cells of which were laden with lipid material. Bioassays for sodium-retaining

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*In this connection it is of considerable interest that within a period of six weeks from the time of the published report four additional cases,[2-5] all associated with a single adrenal cortical adenoma, have been recognized. Removal of the adenoma has resulted in cure in three, and the fourth was recognized in retrospect, having come to autopsy.

corticoid were carried out on extracts of the tumor tissue. Calculations based upon the assay procedure give values for aldosterone which are 75 to 150 times greater per gram of tissue than that existing in beef adrenal and 10 to 20 times greater than that found in hog adrenal gland.

b. *Biopsy of left adrenal:* Gross examination of the gland at the time of surgery suggested mild hypoplasia. Wedge biopsy exhibited mild thinning of the cortex. Microscopically the atrophy was limited to the zona fasciculata which was about one-half as wide as normal. The zona glomerulosa appeared to be slightly wider than normal. There was certainly no suggestion of atrophy of this zone. The zona reticularis appeared normal.

Comment: Atrophy of the zona fasciculata of the gland contralateral to the tumor suggests that the site of formation of aldosterone is the zona fasciculata. Our previous data[1] indicate that there was no depression of the ordinary, conventional, endogenous ACTH production. It would appear, then, that a mechanism other than the conventional ACTH-adrenal axis exists which is capable of exciting or depressing aldosterone production independently of the other secretory activities of the adrenal cortex.

c. *Muscle biopsies:* Three large biopsies from three different sites were obtained. Analyses were done for their content of sodium, potassium, magnesium, water, nitrogen, and phosphate. Whether expressed as dry or wet weight or in terms of ratios to nitrogen, all specimens indicate a great excess of intracellular sodium and marked decrease in the amount of intracellular potassium.

d. *Renal biopsies:* Severe arteriolosclerosis is evident in all sections. A major lesion is seen in the renal tubules where a diffuse vacuolar change is observed in the tubular epithelium. These vacuoles do not take up fat or glycogen stains. This is, therefore, regarded as hydropic degeneration. In some areas this tubular lesion has progressed to necrosis. A few areas of calcification of renal parenchyma are observed.

Comment: It seems likely that the major cause of the tubular lesion is chronic hypokalemia. The associated alkalosis may have been responsible in part for the renal calcinosis. The severe arteriolar lesion is undoubtedly related to excessive activity of sodium-retaining steroid.

2. *Metabolic Changes Following Removal of the Tumor.*—

a. *Sodium and potassium:* Within one day there began a large diuresis of sodium and sharp retention of potassium. Ninety per cent of this exchange was completed during the first ten postoperative days. Hypernatremia and hypokalemia disappeared by the sixth postoperative day and serum values for sodium and potassium have remained normal since that time. Blood pH fell gradually from 7.55 on the morning of operation to 7.42 on the twelfth postoperative day and has remained normal since. Sweat sodium which had averaged 9 meq. per liter for the fourteen days before operation was 45 meq. per liter on the eighth postoperative day and since then has fluctuated within normal limits.

b. *Renal status:* Proteinuria disappeared on the sixth postoperative day and has not appeared since (ninety days). Polyuria and polydipsia, which were very prominent symptoms, disappeared on the fifth postoperative day despite

the fact that renal concentrating ability had not yet improved. Disappearance of these symptoms correlates so well with the return to normal of the previous hypernatremia that we are inclined to believe that the polydipsia was primary and due to hypernatremia in the presence of a normally functioning hypothalamic thirst center. Renal concentrating ability is improving but is not yet normal. This, however, might be expected in view of the severity of the tubular lesion described. Blood pressure which had averaged 170/100 preoperatively began to fall gradually on the twelfth postoperative day, reaching 120/70 on the eighteenth day. It has fluctuated between 130/80 and 110/70 since that time. Bioassays for urinary sodium-retaining corticoid now give normal values. Daily determinations for 17 hydroxycorticoids and 17 ketosteroids continue to be normal as they were in the preoperative period.

c. *Symptomatic changes:* The patient is now entirely asymptomatic. The disturbing polydipsia, polyuria, and nocturia have gone. Muscular weakness has disappeared completely, as has symptomatic tetany. However, weakly positive Chvostek and Trousseau signs can still be elicited, for reasons which are not yet clear (magnesium studies carried on during the past five months have not yet been completed). The patient states that in retrospect she is now aware that her "thinking processes" were greatly impaired before her operation. No objective change, however, can be observed.

d. *Administration of aldosterone** (Feb. 6-9, 1955): During February 6, 7, and 8 the patient was given a continuous intravenous drip which contained 1.0 mg. of aldosterone per twenty-four-hour period. On February 9 she was given 0.5 mg. of aldosterone intravenously and 0.4 mg. intramuscularly. During this period all of the biochemical indices moved in the direction of the preoperative abnormality but quantitatively the changes were much less intense. This is highly suggestive that the patient's tumor had been secreting in excess of 1 mg. per day of aldosterone. In support of this view is the fact that bioassays of the urine for sodium-retaining corticoid showed no increase above normal values during this period of aldosterone administration.

COMMENT

The syndrome of primary aldosteronism seems now to be clearly established. Within a period of six weeks from the initial published report four additional cases typical of this condition have been recognized by others[2-5] and in each one of these cases an adrenal cortical adenoma has been found. Of the total of five cases, one patient died and the diagnosis was made at autopsy.[2] The remaining four patients have been cured by surgical removal of the adenoma. The rapidity with which these patients have been recognized as being characteristic of this syndrome suggests that the condition is more common than previously suspected.

The case of Wyngaarden and associates[6] referred to in our original report showed no adenoma at autopsy. This suggests that the syndrome can occur without adrenal tumor and that subtotal adrenalectomy should be considered

*We are greatly indebted to Dr. Harold Mason, Rochester, Minn., Dr. Harold Hallman, The Upjohn Company, Kalamazoo, Mich., and Dr. Robert Gaunt, Ciba, Inc., Summit, N. J., for contributing the aldosterone used in this study.

when no tumor is found. Since the bioassay procedure for urinary aldosterone does not lend itself to broad clinical use, it is suggested that serum potassium and bicarbonate be done routinely as a screening procedure on all hypertensive patients. In this way some of these individuals may be recognized earlier than they would be otherwise. In the presence of hypokalemic alkalosis, unexplained by other conditions, such individuals should be subjected to adrenal surgery.

The data upon which this brief progress report is based will be published in full.[7]

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